A STUDY OF THE BIOCHEMICAL CHANGES INDUCED IN A NORADRENERGIC SYSTEM IN BRAIN BY ELECTRICAL STIMULATION AND LESIONS OF THIS SYSTEM

by

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Statement in terms of Ph.D. regulation 2.4.15 of the University of Edinburgh.

The work presented in this thesis has been composed by myself with two exceptions.

a) Electrolytic ablation of the locus coeruleus was performed by Drs. G. W. Arbuthnott and T. J. Crow and Miss J. Christie of the University of Aberdeen, in a joint experiment in which the post operative care, dissection and biochemical estimations were performed by myself.

b) Chronic implantation of stimulating electrodes and the training of rats to self stimulate was performed by Miss J. Anlezark, Drs. G. W. Arbuthnott and T. J. Crow of the University of Aberdeen in a joint experiment in which biochemical estimations were performed by myself.

Signed 

(D. S. Walter B.Sc.)

Statement in terms of Ph.D. regulation 2.4.11 of the University of Edinburgh.

Some of the results described in this thesis have been presented as follows.

1. "The effect of Electrical Stimulation of the Locus Coeruleus on the Endogenous Concentration of 4-hydroxy-3 methoxy-phenylethylene glycol in Rat Brain"
   D. S. Walter and D. Eccleston
   Biochemical Journal 128 (1972) 85-86P.

2. "Increase of noradrenaline metabolism following electrical stimulation of the locus coeruleus in the rat"
   D. S. Walter and D. Eccleston
   J. of Neurochem. in press.

3. "The effect of unilateral and bilateral lesions in the locus coeruleus on the levels of 3-methoxy 4-hydroxy-phenylglycol (MHPG) in Neocortex"
   G. W. Arbuthnott, J. E. Christie, T. J. Crow,
   D. Eccleston and D. S. Walter
   Experientia 29 (1973) 52.

4. "Intracranial self-stimulation and noradrenaline metabolism in cortex"
   G. M. Anlezark, G. W. Arbuthnott, T. J. Crow,
   D. Eccleston and D. S. Walter
   Brit. J. Pharmacol 47 (1973) 645P.

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ABBREVIATIONS

A	Adrenaline
acetyl HFB-HMPG	acetyl-heptafluorobutyryl-HMPG
acetyl TFA-HMPG	acetyl-trifluoracetyl-HMPG
acetyl TFA-DHPG	acetyl-trifluoracetyl-DHPG
AMP	Adenosine monophosphate
(AP)	Anterior-posterior stereotaxic co-ordinate
ATP	Adenosine triphosphate
Ci	Curie
COMT	Catechol O-methyl transferase
CSF	Cerebro-spinal fluid
cyclic AMP	Adenosine 3'-5' phosphate
DA	Dopamine
DHMA	3:4 dihydroxymandelic acid
DHPG	3:4 dihydroxyphenyl glycol
DHPG-SO_4	3:4 dihydroxyphenyl glycol sulphate
DOPA	3:4 dihydroxyphenylalanine
D3O	See Table 3 for derivation as for H3O
ECD	Electron capture detector
FAD	Flavin adenine dinucleotide
GLC	Gas liquid chromatography
H CH	Hexachlorocyclohexane
5-HIAA	5-hydroxyindole acetic acid
HFB-acid	Heptafluorobutyric acid
HFB-anhydride	Heptafluorobutyric anhydride
H3O	See Table 3 for derivation
HMPG	4-hydroxy 3 methoxy phenyl glycol

HMPG-SO_4	4-hydroxy 3 methoxy phenyl glycol sulphate
HVA	4-hydroxy 3 methoxy phenylacetic acid
5-HT	5-hydroxytryptamine
(L)	Lateral stereotaxic co-ordinate
LC	locus coeruleus
LSD	lysergic acid diethylamide
MAO	Monoamine oxidase
α MMT	alphamethyl metatyrosine
NA	Noradrenaline
OD	Outside diameter
P	Probability
PNMT	Phenylethanolamine N-methyl transferase
POPOP	p-bis [2-(5 phenyloxazolyl)] -benzene
PPO	2:5 diphenyl oxazole
REM Sleep	Rapid eye movement sleep
SD	Standard deviation
SWS	Slow wave sleep
TFA anhydride	Trifluoracetic anhydride
TLC	Thin layer chromatography
(V)	Vertical stereotaxic co-ordinate
VMA	4-hydroxy 3-methoxy mandelic acid

SUMMARY

The work of this thesis is divided into two sections. Section I describes the development of methods for the quantitative estimation in rat brain of 4-hydroxy 3 methoxy-phenyl glycol (HMPG), 3:4 dihydroxy-phenylglycol (DHPG) and their conjugates (HMPG-SO_4 and DHPG-SO_4).

The first part of this section tests the possibility of adapting the fluorimetric method of Antun et al (1971) for the quantitative estimation of HMPG in rat brain. This method was found to be unsuitable because of its limited sensitivity which was further reduced due to interfering fluorescence from contaminants introduced with the addition of the crude enzyme preparation 'Helicase', the latter being necessary for the hydrolysis of the glycol conjugates.

The remainder of the section describes the successful application of methods to make acetyl, trifluoracetyl derivatives of HMPG and DHPG, which derivatives were suitable for quantitative analysis using gas liquid chromatography with electron capture.

The mean concentration of total (free + conjugated) HMPG found in whole brain was 95ng/g and in cerebral cortex was 102 ng/g where about one fifth of the amounts was consistently found in the unconjugated (free) form. This was not the case with DHPG. Only trace amounts of DHPG were found in one or two samples of whole rat brain and cerebral cortex, whereas the concentration of DHPG-SO_4 was equal to the concentration of HMPG-SO_4. Possible reasons to explain the lack of free DHPG are put forward.

Section II describes some effects following the electrophysiological manipulation of a noradrenaline containing neuronal system in rat brain, that having its cell bodies in the nucleus locus coeruleus, which is situated on the floor of the 4th ventricle, and from which projects a discrete bundle of axons, shown to be largely uncrossed, which have their terminals in the cerebral cortex and hippocampal formation.

Working on the hypothesis that any alteration of the activity of this system would cause some change in the endogenous concentration of the major noradrenaline metabolite HMPG (free + conjugated), the nucleus locus coeruleus was

a) electrically stimulated on one side and the effect on metabolism in left and right cortex examined.

b) electrolytically ablated and after a period of 3 weeks, the effect on metabolism in left and right cortex examined.

Ablation experiments confirmed observations that the pathway was uncrossed since a fall in HMPG concentration occurred only on the side of the lesion, whereas stimulation suggested that either there was crossing over of some neurones or that simultaneous stimulation of both nuclei was occurring, since stimulation, although tending to cause a larger increase on the stimulated side nearly always gave an increase in the contralateral cortex.

In addition to these experiments, work is described which shows the relation between conscious self stimulation

behaviour in rats with chronically implanted electrodes with their tips in the region of the locus coeruleus, and the metabolism of noradrenaline in rat cerebral cortex. During conscious self stimulation, the HMPG concentration in cortex rose on the stimulated side, but also on the contralateral side. Because the stimulus was by design, quite localised and specific to the region just around the electrode tip, this ruled out the possibility of co-stimulation of the opposite nucleus locus coeruleus by spread of stimulation current. However, in view of work describing a medial pathway from locus coeruleus, the possibility exists that the contralateral effect could occur as a result of the activation of such a pathway during stimulation. This and the relevance of the results of stimulation and ablation are discussed.

SECTION I

DEVELOPMENT OF METHODS FOR THE QUANTITATIVE ESTIMATION OF HMPG AND DHPG IN RAT BRAIN

INTRODUCTION

It is over seventy years since the first observation by Lewandowsky (1899) that suprarenal extract could mimic the actions produced by stimulating sympathetic nerves. Two years later, Langley (1901) repeated this work and concluded that there was a good correlation between electrical stimulation of the sympathetic system and stimulation using the extract from the suprarenal gland, but could not explain why the latter should persist after denervation. It was Elliot (1905), a student of Langley, who, in addition to confirming previous observations, put forward a hypothesis to explain the mode of action of the active principal in the extract. He suggested that "a mechanism developed out of the muscle cell, in response to it's union with the synapsing sympathetic fibre, the function of which is to receive and transform the nervous impulse. Adrenaline might then be the chemical stimulant liberated on each occasion when the impulse arrives at the periphery." An essentially similar idea to this was proposed at a later stage by Dixon and Hamill (1909), but the first experimental confirmation of the theory of chemical transmission did not come until 1921 when Loewi showed that electrical stimulation of the vagus nerve supply of an isolated frog's heart, perfused with Ringer's solution, caused the release of an active principal into the perfusate, which when the perfusate was passed through a second frog's heart caused inhibitory effects typical of vagal stimulation. Effects on the second heart could also be obtained

following stimulation of the sympathetic fibres of the first frog's heart, the released chemical in this case having an effect typical of sympathetic stimulation. Later studies showed that the substance released following vagal stimulation was acetylcholine, and that following sympathetic stimulation, an adrenaline-like substance given the name 'Sympathin'. It also became apparent that there was some disparity between the pharmacological action of synthetic adrenaline and 'Sympathin' from various organ extracts, which led Cannon and Rosenbleuth (1933) to postulate the existence of two types of sympathin, E- producing excitatory responses and I - producing inhibitory responses. The chemical nature of these substances was not known, but Bacq (1934) suggested that sympathin E might be noradrenaline and sympathin I, adrenaline. Most of the accumulated evidence, however, pointed to the fact that the chemical transmitter of the sympathetic system was adrenaline, and this view persisted until 1946 when Euler (1946a,b, 1947,1948,1950) reported a whole series of results which showed that the predominant catecholamine in tissue extracts was noradrenaline, based on comparisons he made of the pharmacological actions of tissue extracts with adrenaline and 3:4 dihydroxynorephidrine (the nearest analogue to noradrenaline available at the time) and later with noradrenaline itself, when it was clear that sympathin behaved more like the non N-methylated compounds, dihydroxy-norephidrine and noradrenaline, than synthetic adrenaline. The earlier observation by Blaschko (1939) that noradrenaline

was probably a precursor of rather than a breakdown product of adrenaline gave credence to these results. But, although the presence of noradrenaline was confirmed by other workers in tissues and nerves (Bacq and Fischer 1947, Gaddum and Goodwin 1947), in human urine (Holzt, Credner and Kroneberg 1947) and in extracts of blood vessels (Schmiterlöw 1948) and although it was shown that noradrenaline was released into the blood stream following electrical stimulation of sympathetic nerves (Peart 1949, West 1950, Mann and West 1950,1951, Outschoorn and Vogt 1952, and Outschoorn 1952) it was some time before it could be certain that noradrenaline was the main sympathetic chemical transmitter. During the next decade, there were many reports of the concentrations of noradrenaline and adrenaline in body tissues, measured using either the sensitive fluorescence technique described by Lund (1949) which depends on the formation of a trihydroxyindole oxidation product of the β-hydroxylated catecholamines, or the ethylene diamine condensation method described by Weil-Malherbe and Bone (1952) (although some prior separation of adrenaline and noradrenaline was necessary before using the latter technique). Noradrenaline was found to be present in most tissues in much larger concentrations than adrenaline, and had a distribution which matched that of the sympathetic innervation, these facts being suggestive that noradrenaline was the chemical transmitter of this system. Confirmation of this fact was obtained after the introduction by Falck (1962) of a fluorescence histochemical

technique, which besides revealing that the distribution of noradrenaline in situ was correlated with the distribution of sympathetic innervation, was capable of distinguishing noradrenaline from adrenaline. Vogt in 1954 showed that noradrenaline was present in cat brain in relatively large amounts and did not have a uniform distribution, high concentrations being found in the hypothalamus and the brain stem and low concentrations in the cerebellum and cerebral cortex. This suggested therefore, that noradrenaline might have a function in brain more than just as a chemical transmitter associated with the sympathetic innervation of the blood vessels of the brain, since the latter had a uniform distribution. More weight was given to this suggestion following the demonstration by Bertler and Rosengren (1959a) that there was no change in the levels of noradrenaline and dopamine following the removal of the cerebral sympathetic innervation. Bertler and Rosengren (1959b) also showed that both catecholamines had an unequal distribution in the brain, but with a different pattern, the highest concentrations of dopamine being found in the corpus striatum. In the following years, workers demonstrated, using the fluorescence histochemical technique of Falck (1962), the presence of discrete groups of catecholamine nerve cell bodies located in the brain stem and mesencephalon (Carlsson et al. 1962, Dahlström and Fuxe, 1964) and networks of nerve terminals in most regions of brain (Fuxe 1965) with the eventual tracing, in part, of the neuronal pathways from the cell bodies to the nerve

terminals (Anden et al. 1964, Ungerstedt 1971). This aspect of the work is discussed more fully in the introduction to Section 2.

The biosynthesis of catecholamines in the central nervous system and the periphery is summarized by the pathway in Fig.1. which shows that the metabolic sequence starts with the hydroxylation of tyrosine to give dihydroxyphenylalanine (DOPA), followed by the decarboxylation of DOPA with aromatic amino acid decarboxylase to form dopamine (DA). DA is then hydroxylated in the aliphatic β-carbon position forming noradrenaline (NA), and adrenaline (A) formed by the N-methylation of NA. This exact metabolic sequence was suggested as a possibility by Blaschko in 1939 following the discovery of an enzyme which would decarboxylate DOPA (Holtz, Heise and Ludtke 1938). This enzyme, called then dopa decarboxylase, but since it is not specific for DOPA, now called aromatic amino acid decarboxylase, was found to have a wide distribution in both peripheral tissues and brain, and in brain was distributed closely with areas having a high catecholamine content (Holtz and Westermann 1956, Bertler and Rosengren 1959b).

The first enzyme in the reaction sequence, tyrosine hydroxylase, a membrane bound enzyme demonstrated by Nagatsu et al. (1964) in the adrenal medulla, in tissue rich in sympathetic innervation and in the brain, has recently been shown to be specifically associated with either catecholamine-containing nerve cell bodies and terminals, where the enzyme appears to be particulate, or

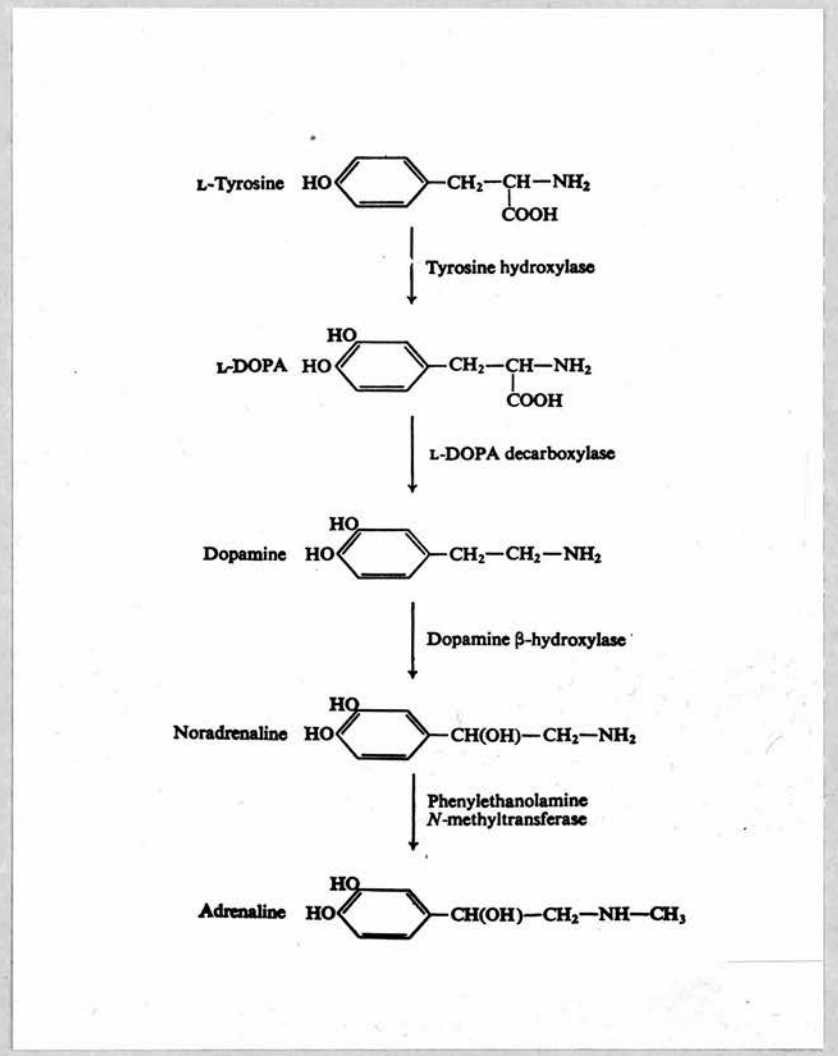

FIG.1 Figure showing the major biosynthetic pathway of dopamine and noradrenaline.

with the cells of the adrenal medulla, where it appears to be soluble. The actual subcellular location of the enzyme in nerve terminals is, however, still unclear since Fahn et al. (1969) showed that it was intimately connected with the synaptic vesicle subfraction, whereas Stjärne and Lishajko (1967) found the enzyme in the soluble subfraction along with aromatic amino acid decarboxylase, which enzymes were distributed quite differently from synaptic vesicle bound dopamine β-hydroxylase. Tyrosine hydroxylase is the rate limiting enzyme in NA biosynthesis, where end product, feed back inhibitionis presumed to pay a role in the control of synthesis rate (Levitt et al.1965). The enzyme is thought to exist in an inactive oxidised form, which is activated by interaction with a reduced pteridine cofactor, the reduced tyrosine hydroxylase combining with oxygen and the substrate to complete the enzymic hydroxylation. It is therefore possible that regulation of synthesis rate is influenced by the availability of the reduced pteridine cofactor, the latter being regenerated by the further reduction of the oxidised cofactor with the enzyme, dihydroxypteridine reductase (Ikeda et al.1966).

The enzyme which converts DA to NA, dopamine β-hydroxylase is a mixed function oxidase containing copper and needing molecular oxygen and a reducing agent as cofactors, ascorbic acid probably serving as the latter (Friedman and Kaufman 1965). This enzyme has been more localised than either tyrosine hydroxylase or aromatic amino acid decarboxylase, being found only associated with subcellular fractions

rich in synaptic vesicles (Stjärne and Lishajko 1967) in the membranes of which it is presumed to be bound.

Although the three enzymes needed for the biosynthesis of NA from tyrosine are present in catecholamine nerve cells of the sympathetic nervous system and in brain, this is not true for phenylethanolamine N-methyl transferase (PNMT), the enzyme responsible for the N-methylation of NA to form A. As well as in the adrenal medulla, where it exists in large amounts, and in the heart (Axelrod 1962) this enzyme has been demonstrated in the brain, only in certain nerve cells of the olfactory bulb and tubercle (Milhaud and Glowinski 1962, McGeer and McGeer 1964, Pohorecky et al.1969) although endogenous A has yet to be demonstrated in these nerve cells. The enzyme has a co-factor requirement for S-adenosyl methionine and Mg^{++} ions, in common with all methyl transferases.

Since all three enzymes for the synthesis of NA have been shown to be associated with the catecholamine containing nerve cells, much work has been made to discover the exact subcellular location of synthesis. It now seems certain that NA can be synthesised either in nerve cell bodies (Carlsson 1966, Dahlstrom and Fuxe 1964) or in nerve terminals (Levitt et al.1965) the hydroxylation of tyrosine and the decarboxylation of DOPA probably occurring in the cell sap, and the hydroxylation of DA occurring inside the synaptic vesicles, following its active uptake. Vesicles synthesised in the cell body are transported down the axon to the nerve terminals (Van Breeman 1958, Dahlstrom and

Fuxe 1964) where they supplement the vesicle store, replacing vesicles lost during synaptic transmission.

The metabolic degradation of NA in the brain, in vivo, has been shown by following the fate of radioactively labelled amine, injected into the lateral ventricle of the rat brain. A whole range of metabolites are demonstrable which fall into three main groups, the 4-hydroxy 3-methoxy-phenyl metabolites, the 3:4 dihydroxyphenyl metabolites, and the sulphate or glucuronide conjugates of either of the two groups. The various catabolic pathways are summarised in Fig. 2.

The physical and chemical properties of two of the enzymes involved in NA catabolism, monoamine oxidase (MAO) and catechol O-methyl transferase (COMT) are well understood, but their relative importance in the termination of the action of NA is still unclear.

MAO, described by Hare in 1928 as an enzyme which would oxidise tyramine, was found to be active also in the oxidative deamination of catecholamines (Blaschko et al.1937). It is however not specific for catecholamines and has a wide distribution in most animal tissues. It is mitochondrial bound (Hawkins 1952, De Lores Arnaiz and De Robertis 1962) and a flavoprotein containing 2 moles of FAD per mole of enzyme (Yashnobu 1968). Cytochrome C1 and copper are also cofactors. Blaschko's group also showed that MAO has a high affinity for phenylethylamine derivatives which lack a β-hydroxyl substituent and which have mono-

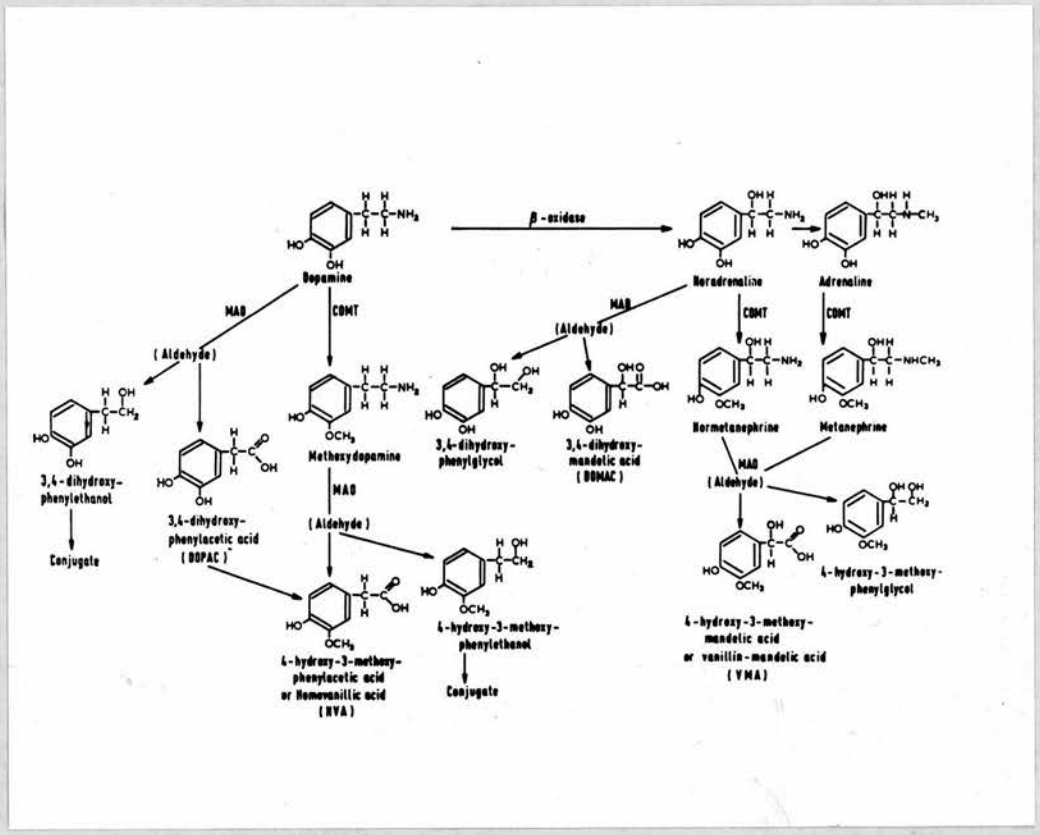

FIG.2 Figure showing the various pathways for the catabolism of dopamine and noradrenaline.

phenolic or catechol substituents in the aromatic ring. (Blaschko et al. 1937; Pratesi and Blaschko 1959; Blaschko 1952).

Axelrod in 1957 discovered an enzyme (COMT) which caused the transfer of a methyl group from S-adenosyl methionine to the 3-OH phenyl group of a catechol. This enzyme, found in most cells is bery abundant in liver and kidney (Axelrod et al. 1959) and has been demonstrated in brain, where it is thought to be a soluble enzyme.

Little is described about the enzymes which convert the aldehydes, fromed from the oxidative deamination of the amines by MAO, to either the acid or the neutral metabolites. Since the intermediate aldehyde metabolites have not been demonstrated, the enzymes causing the further reduction or oxidation are probably intimately coupled with MAO, and hence, probably bound to mitochondria. Breese et al.(1969), however, have shown that the presence of a β -OH group will facilitate the formation of a neutral metabolite, whereas the lack will give rise to an acid metabolite, although no real reason for this phenomenon has been put forward.

The ability of tissues to form conjugates of phenolic compounds has been well documented, and the sulphate esters of NA metabolites are probably formed using the intra-cellular located enzyme system described by Roy (1971) where a two stage process is thought to be involved (Fig.3). The

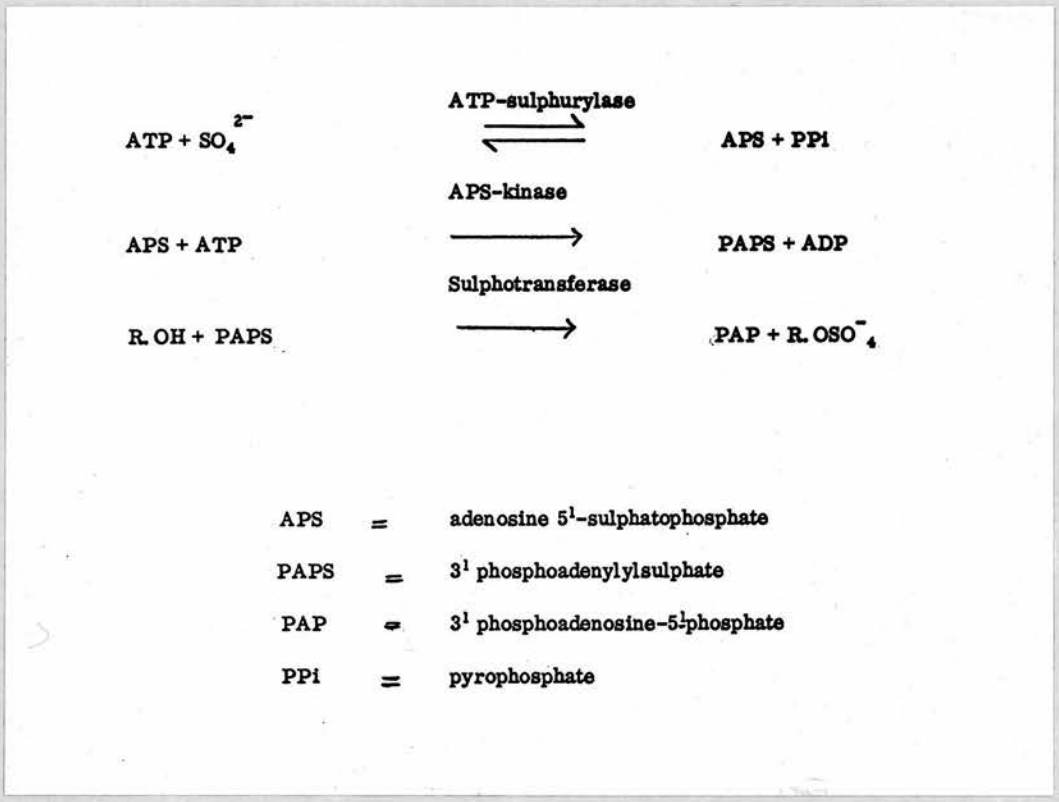

FIG. 3 Figure showing the various stages of sulphation of phenolic compounds in the liver. Similar mechanisms are thought to occur in brain.

first stage is the activation of sulphate, itself a two stage process involving the addition of a sulphate group to AMP and the phosphorylation of the ribose 3-OH group using ATP. The second stage is the transfer of the sulphate group to the recipient phenol group.

Since NA turnover in the sympathetic nervous system was found to be correlated with neuronal activity, (Hertting and Axelrod 1961) attempts were made to measure the turnover of the amine in brain as a possible clue to it's functional importance. Since NA and other amines are restricted from entry into brain, by the blood-brain barrier (Weil Malherbe et al. 1959) other ways of measuring the turnover were devised. Three methods give similar results for the turnover of NA in rat brain. These are

(1) measurement of the rate of disappearance of endogenous NA after synthesis blockade using a tyrosine hydroxylase inhibitor

(2) measurement of the rate of disappearance of (a) (^{3}H)-NA injected into the lateral ventricle or (b) (^{3}H)-NA formed from the precursors (^{3}H)-DA or (^{3}H) DOPA injected into the lateral ventricle and

(3) measurement of the rate of disappearance of radioactively labelled NA, synthesised from labelled tyrosine injected intravenously.

Using these methods it was shown that the turnover rate of NA, as with the endogenous concentration of NA, was different in various brain areas, but that the turnover rate was highest in areas of low concentration of amine, and vice versa (Udenfriend et al. 1963, Burack and Draskoszy 1964, Iversen and Glowinski 1966).

Another way of estimating the turnover of NA in brain would be to look at the changes in concentrations of its metabolites measured either as a change in the specific activity of the metabolite after the labelling of the endogenous amine stores, or, depending on the efficiency of the metabolite transport mechanism, simply by an accumulation of the metabolite. Two problems arise, firstly, which is the major metabolite of NA in brain? and secondly, does the change in level of this metabolite reflect the turnover of the amine? Regarding the first problem, the major metabolite of NA in brain has been shown, by analysis of the radioactivity after an intraventricular injection of labelled amine, to be 4-hydroxy 3-methoxyphenyl glycol (HMPG) or its sulphate ester (HMPG-SO_4). Mannarino et al. (1963) injected (^{14}C)-NA into the cat lateral ventricle and looked at the metabolites formed in the cerebellum, cerebral cortex and urine. The major metabolite in brain from the exogenous (^{14}C)-NA was HMPG whereas in urine it was the conjugated glycol. Schanberg et al. (1968b), however, showed that in rat brain, the major metabolite of both (^{3}H)-normetanephrine and (^{14}C)-NA injected into the lateral ventricle, was conjugated HMPG, the radioactivity in the combined VMA and free HMPG fraction being only a tenth of that of the conjugate fraction. They further showed that the conjugate was probably a sulphate ester, since hydrolysis to (free) HMPG only occurred with a sulphatase preparation, whilst a β-glucuronidase preparation was ineffective. Only small, insignificant amounts of the 3:4 dihydroxyphenyl metabolites of NA were detectable in each case.

Supporting the idea that the neutral metabolite of NA, HMPG and its sulphate ester, HMPG-SO_4 are more important.

than the acid metabolite VMA, was the demonstration by Schanberg et al (1968a) of endogenous HMPG in brain of a number of species, and by Sharman (1969) in mouse, cat and dog hypothalamus, whereas endogenous VMA has yet to be demonstrated in brain. (Chase et al 1971). HMPG and HMPG-SO_4 have also been demonstrated in CSF of animals (Schanberg et al 1968a) and humans (Maas et al 1968, Wilk et al 1971, Bond 1972).

Working on the hypothesis that HMPG and HMPG-SO_4 are important metabolites of NA in brain, the work in this section describes the development of a sensitive and accurate technique for their estimation. This technique is then modified to permit the simultaneous estimation of 3:4 dihydroxyphenylglycol [DHPG] and its sulphate ester, in order to determine their relative importance as metabolites.

Section II describes the effect of electrical stimulation or electrolytic ablation of a NA-containing neuronal system in brain on the metabolite level in whole brain and more particularly in cerebral cortex, in an attempt to answer the question as to whether the level of these metabolites reflects the turnover of NA in brain.

METHODS

Brain removal and dissection

Rats were killed by breaking the neck . The brain was removed from the skull and placed on a glass plate. With the dorsal surface of the brain uppermost, the cerebellum was gently eased from its position overlying the brainstem. The brain was then bisected with a vertical cut in the mid line, the two halves placed flat on the glass plate and the cerebral cortex and hippocampus gently peeled from the underlying grey matter with a microspatula. Corpus striatum, which after this procedure still remained attached to the cerebral cortex/hippocampus, was then carefully removed.

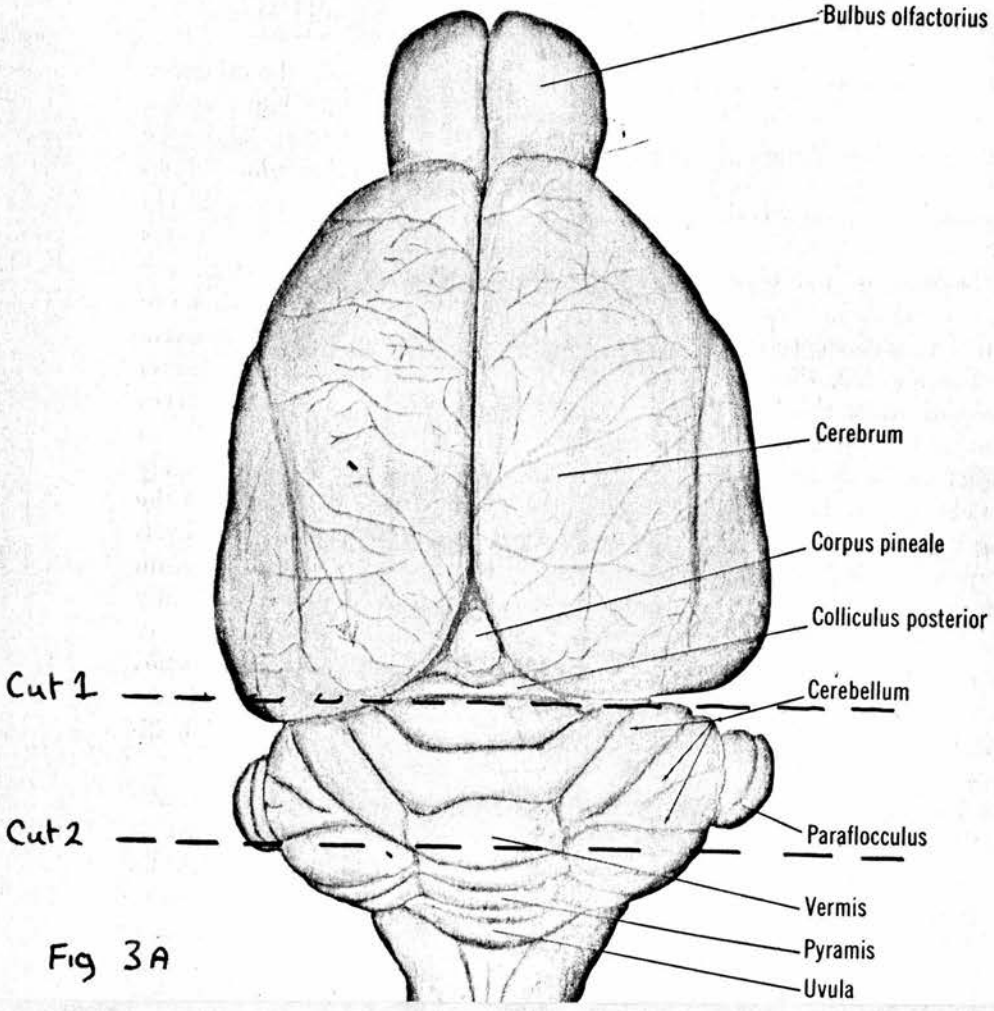

Fig 3A

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Where possible, the analysis of the noradrenaline metabolites was performed in fresh tissue, but in large experiments which precluded this, whole brain or parts of brain were placed in stoppered vials and kept at -20°C for periods of up to 4 weeks, without there being any alteration in the metabolite levels, as judged by the values for control rats at various times of storage (table 1).

Homogenisation and precipitation of brain protein and lipid

Two methods for the precipitation of brain protein and lipid were used. a) Perchloric acid

b) Boiling water/petroleum ether.

(a) Perchloric acid method

Weighed samples of brain tissue were homogenised in 5 ml of 0.4M perchloric acid at 5°C with an all glass homogeniser, the homogeniser washed with a further 1 ml of 0.4M perchloric acid, the homogenate and wash combined, and centrifuged at 10,000g for 15 min. The clear supernatant was adjusted to between pH 4 and pH 5 with 5N and 0.1N KOH by glass electrode. To obtain maximum precipitation of potassium perchlorate the solution remained at 5°C for 15 min prior to low speed centrifugation at 3000g for 10 min at 5°C.

Perchloric acid is a very efficient precipitant of brain protein and lipid, but it was not a very useful method when the subsequent quantitative estimation of HMPG was to be made using the fluorimetric method of Antun et al. (1971), since it was found that both perchloric acid and

TABLE 1 The concentration of total HMPG in rat cerebral cortex after various stages of storage, showing that there was no significant change in glycol levels with tissue kept at -20°C for up to 4 weeks. (NS = not significantly different from control values).

Concentration of Total HMPG in Rat Cerebral Cortex ng/g				
	Fresh Tissue	Weeks at -20°C		
		2	3	4
	94	171	82	75
	117	57	75	82
	130	118	89	82
	81	127	114	118
mean ± SD	106 ± 19	118 ± 40	90 ± 15	89 ± 17
		t = 0.49 NS	t = 1.11 NS	t = 1.10 NS

potassium hydroxide contributed substantially to the blank value of this fluorimetric method, making the estimation of small amounts of HMPG difficult. The following method, therefore, was devised in order to produce a clean blank for the fluorimetric analysis of HMPG.

(b) Boiling water/petroleum ether method

Weighed samples of brain tissue were homogenised in 8 ml of hot distilled water in an all glass homogeniser. The homogenate was quantitatively transferred to a test tube in a water bath at 100°C and incubated for 10 min. The flocculent precipitate of brain protein was removed by low speed centrifugation at 3000g for 15 min at 5°C and the supernatant transferred to a test tube containing 10 ml of petroleum ether (40°-60°C boiling range). The tubes were stoppered and shaken for 3 min to remove lipid, after which the tubes were centrifuged at 3000g for 15 min to separate the layers. The petroleum ether (upper) layer containing extracted lipid was then removed by suction through a pasteur pipette at the water pump.

This method gave a very good blank, of the same order as that produced by distilled water.

Ascorbic acid has been included in homogenisation procedures to prevent oxidation of labile substances, but in this system the only effect it was found to have was that of slightly increasing the fluorescence blank values, without improving the recovery of added HMPG taken through the method (Table 2). It was therefore not added in subsequent work. Table 2 also shows the usefulness of petroleum

TABLE 2 Table showing the effect of the inclusion of ascorbic acid in the homogenisation medium, and the use of petroleum ether extraction of the supernatant, on the recovery of the same starting amount of HMPG in distilled water.

Sample		Fluorescence (arbitrary units)		Percentage recovery	Mean
		Actual	Minus Blank		
No ascorbic acid	Blank	5	-		
Extracted with	1	31	26	45	47
petroleum ether	2	33	28	48	
No ascorbic acid	Blank	12	-		
No petroleum	1	22	10	17	19
ether	2	24	12	21	
With ascorbic acid	Blank	6	-		
Extracted with	1	32	26	45	47
petroleum ether	2	35	29	50	
With ascorbic acid	Blank	23	-		
No petroleum	1	35	12	21	28
ether	2	43	20	35	

ether extraction for obtaining clean blanks.

Separation procedures for amines and their metabolites

The success of any method for the purification and quantitative estimation of a substance may depend on the removal of similar substances which might behave similarly in the final analysis. In preliminary experiments, therefore, techniques were developed to separate the amines and their known metabolites.

(a) Removal of amines and amine metabolites

Possibly the most efficient way of removing amines from an aqueous solution is by cation-exchange chromatography. After removal of potassium perchlorate at pH 4.0, the brain supernatant was adjusted to pH 7.5 with KOH, and applied to a 7 cm x 0.7 cm column of 'Amberlite' CG 50 resin, 100-200 mesh in the NH_4^+ form, collecting the effluent. The column was washed with 2ml of 0.2M ammonium acetate buffer, pH 7.5, and this was combined with the effluent. Amines which had been retained, could be removed from the CG 50 resin, eluting with 10 ml of 0.2N HCl.

(b) Removal of acid metabolites

Acid metabolites such as VMA (4-hydroxy 3 methoxy-mandelic acid) and DHMA (3:4 dihydroxymandelic acid), but not the acidic sulphate conjugates of HMPG and DHPG, could be extracted from the CG 50 effluent adjusted to pH 1.0 with HCl, by shaking for 3 min with 3x2 volumes of ethyl acetate. As this technique depends on the suppression of ionization of the carboxylic acid group at the low pH,

the highly charged sulphate conjugates are not extracted since they are completely ionised even at low pH.

(c) Removal of the glycol metabolites

(Free) HMPG and DHPG were extracted from the supernatant adjusted to pH 5.0 with KOH, by shaking with 3x2 volumes of ethyl acetate. The mixture was centrifuged at low speed to separate the layers, the ethyl acetate (upper) layer removed and transferred to a 100 ml round bottom flask. The combined extract was then reduced to dryness in vacuo at 50°C and made up in either 0.5 ml of distilled water, or in 0.2 ml of a mixture of 1:2 dichlorethane and methanol in the proportions of 7:3, depending on the next stage.

(d) Purification of the glycol metabolites

Further purification of the glycol fraction was attempted by chromatography of the resuspended ethyl acetate extract on either a column of 'Sephadex' LH 20 resin or a column of 'Bio-Rad' AG 1x4 anion exchange resin.

1) The use of 'Sephadex' LH 20 resin

'Sephadex' LH 20 resin is a hydrophobic form of 'Sephadex' G 25, designed to be used with organic solvent systems. Its application to the study of catecholamines and their metabolites has been investigated by Anggard et al (1970a, b) who showed a good separation of the metabolites using various mixtures of 1:2 dichlorethane and methanol. The possibility of its use as a preparative method for the glycol metabolites of NA was therefore investigated.

'Sephadex' LH 20 was allowed to swell overnight in a mixture of 1:2 dichlorethane and methanol in the proportions of 7:3, after which the slurry was carefully poured into a 70 cm x 1.1 cm (internal diameter) glass column, to a bed height of 47 cm. The column was filled up with solvent mixture and maintained so during chromatography. Samples were dissolved in 0.2 ml of solvent mixture and carefully applied to the surface of the gel. This was allowed to penetrate the gel, washed with a further 1 ml of solvent mixture and the column filled up. 1.5 ml aliquots of effluent were collected and analysed either for fluorescence by the method of Antun et al (1971) or for radioactivity by scintillation spectrometry.

For fluorescence assay, 0.5 ml of the effluent was blown to dryness with dry N_2 and redissolved in 0.5 ml of distilled water. For scintillation counting, 0.2 ml of the effluent was added to 6.5 ml of a toluene-based scintillant containing 0.425% w/v PPO and 0.0112% w/v POPOP.

In order to determine how the glycol metabolites of NA behaved on the LH 20 column, radioactively labelled HMPG and DHPG were prepared from (^{3}H)-normetanephrine and (^{14}C)-noradrenaline after injection of these amines into the lateral ventricle of the rat brain.

Male albino Wistar rats (120-150g) under light diethyl ether anaesthesia were injected with either (^{3}H)-normetanephrine (20 µCi, specific activity 5.7 Ci/mmol, New England Nuclear), or with (^{14}C)-DL noradrenaline (0.25 µCi, specific activity 27.6 mCi/mmol, Radiochemical Centre, Amersham),

dissolved in 20 µl Merlis solution, according to the technique of Noble et al (1967). One hour after injection, the rats were killed, the brains removed and homogenised in 0.4M perchloric acid to precipitate brain protein and lipid, as described. Amines were removed on a column of 'Amberlite' CG 50 resin, and the acid metabolites extracted from the column effluent at pH 1.0 using ethyl acetate, as described.

Free glycol metabolites were extracted with 3x2 volumes of ethyl acetate after adjusting the pH to 5.0. The ethyl acetate extract was reduced to dryness in vacuo at 50°C and the residue made up in 1 ml of LH 20 column solvent mixture. (The aqueous solution remaining after removal of the amines, the acid metabolites and the free glycols, contained the radioactively labelled conjugated glycol metabolites of NA. These metabolites were separated using paper chromatography, extracted from the chromatograms and the purified metabolites used in studies of the hydrolysis of glycol conjugates using 'Helicase' enzyme preparation, (described later).

0.2 ml of the resuspended extract containing the neutral metabolites including HMPG and DHPG, was applied to the LH 20 column, and the effluent in each case monitored for radioactivity. Figures 4 and 5 show the LH 20 effluent profiles of radioactivity after the intraventricular injection of (^{3}H)-normetanephrine and (^{14}C)-DL-noradrenaline respectively. Chromatography of the extract after intraventricular (^{3}H)-normetanephrine resulted in a

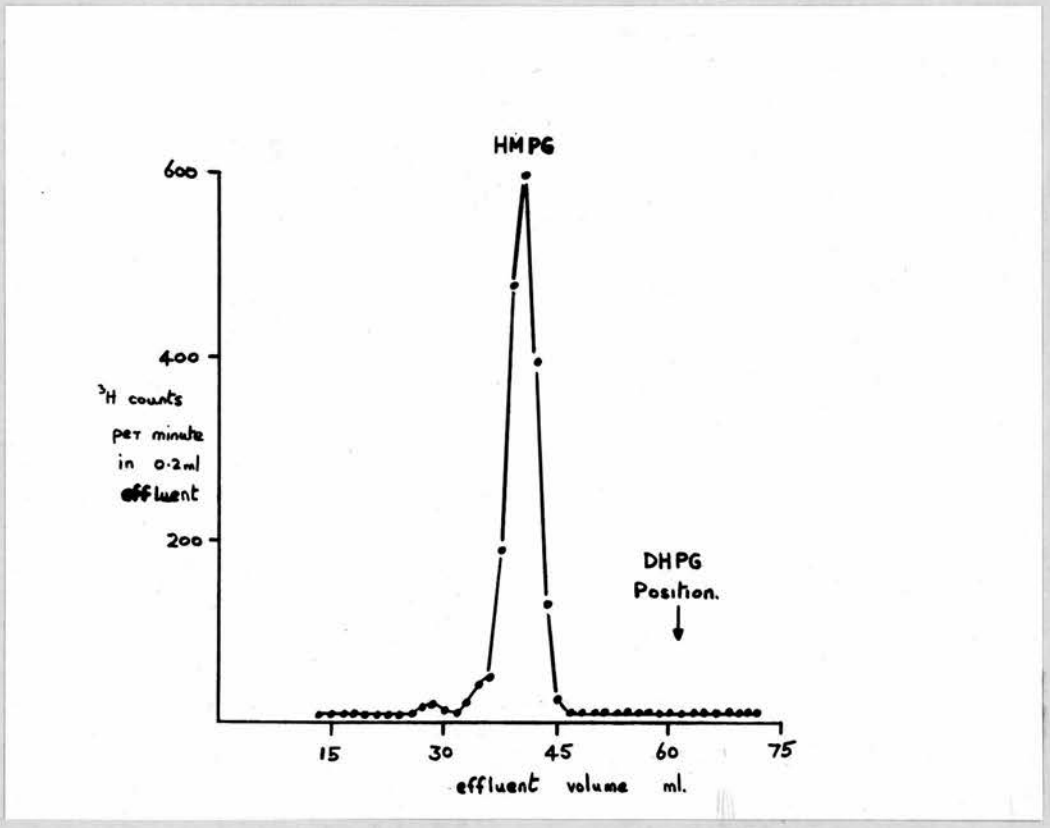

FIG.4 Chromatography, on a column of 'Sephadex' LH20, of the pH 5.0 ethyl acetate extract from a rat brain supernatant following the intraventricular injection of (^{3}H)-normetanephrine, showing a single peak of radioactivity at 41 ml due to (^{3}H)-HMPG. Column solvent system, 1:2 dichlorethane/methanol 7:3.

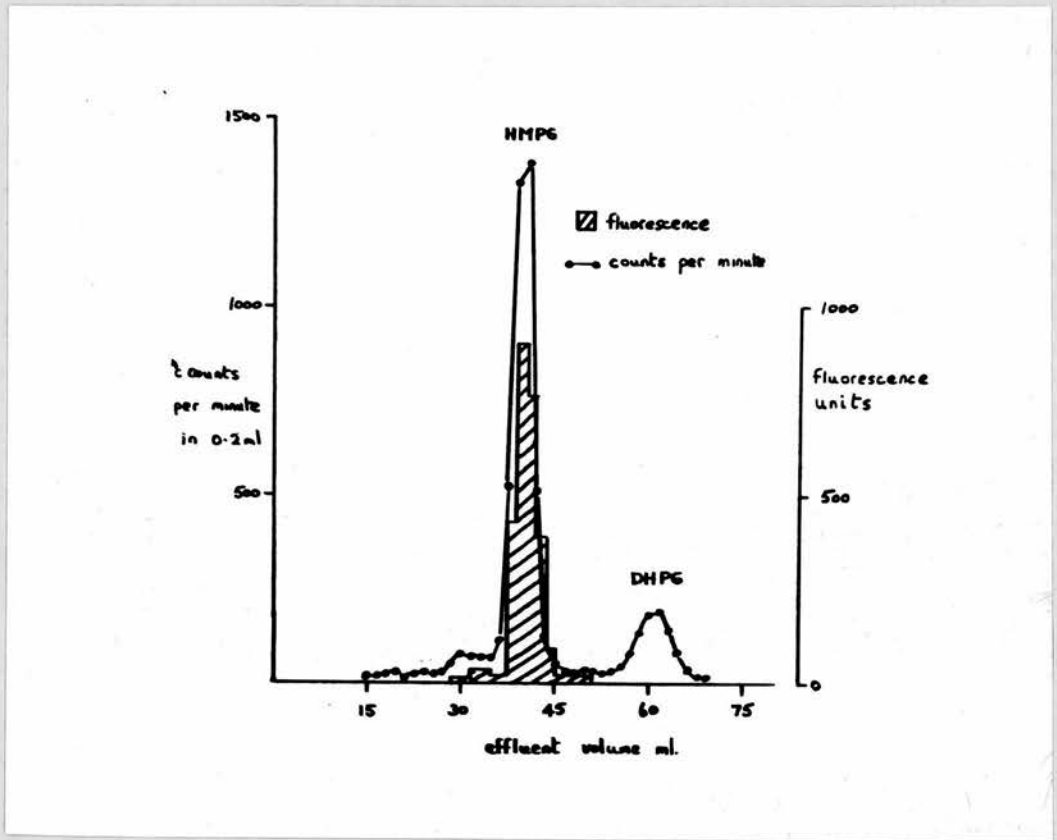

FIG.5 Chromatography, on a column of 'Sephadex' LH20, of the pH 5.0 ethyl acetate extract from a rat brain supernatant following the intraventricular injection of (^{14}C) DL-noradrenaline, showing two radioactive peaks at 41 ml and 62 ml due to (^{14}C)-HMPG and (^{14}C)-DHPG respectively. The shaded histogram shows the chromatography of HMPG, monitored by the fluorimetric method of Antun et al (1971).

single peak of radioactivity which appeared after 41 ml of effluent, whereas the extract from the (^{14}C)-DL-noradrenaline injection contained two peaks of radioactivity, a large peak after 41 ml and a smaller peak after 62 ml of effluent, with a ratio of 6:1. This immediately suggested that the radioactivity after 41 ml was due to HMPG since it appeared in both extracts, and that the peak after 62 ml was due to DHPG. Thin layer chromatography (TLC) of these radioactive peaks was therefore carried out for positive identification.

Effluent from peak tubes was combined and reduced to dryness, the residue resuspended in 10 µl of ethanol and spotted onto a cellulose thin layer plate along with marker amounts of VMA, DHMA, HMPG and DHPG. The developing solvent system used was Chloroform, methanol and N ammonium hydroxide, (12:7:1), developing time 2 hours. The plates were then dried, sprayed, the spots scraped from the glass plate and placed in scintillation vials as described in the section on TLC. Fig. 6 shows the separation of the various metabolites obtained, with the level of radioactivity present in each spot. Radioactivity from the LH 20 peak at 41 ml was isographic with the HMPG spot, whereas only 60% of the radioactivity from the peak at 62 ml was isographic with the DHPG spot. The occurrence of radioactivity in the VMA and DHMA spots was probably due to slight breakdown of DHPG at the alkaline pH. These results indicate therefore that the LH 20 column may be a useful purification step for the separation and quantitative estimation of the glycol metabolites of NA from rat brain.

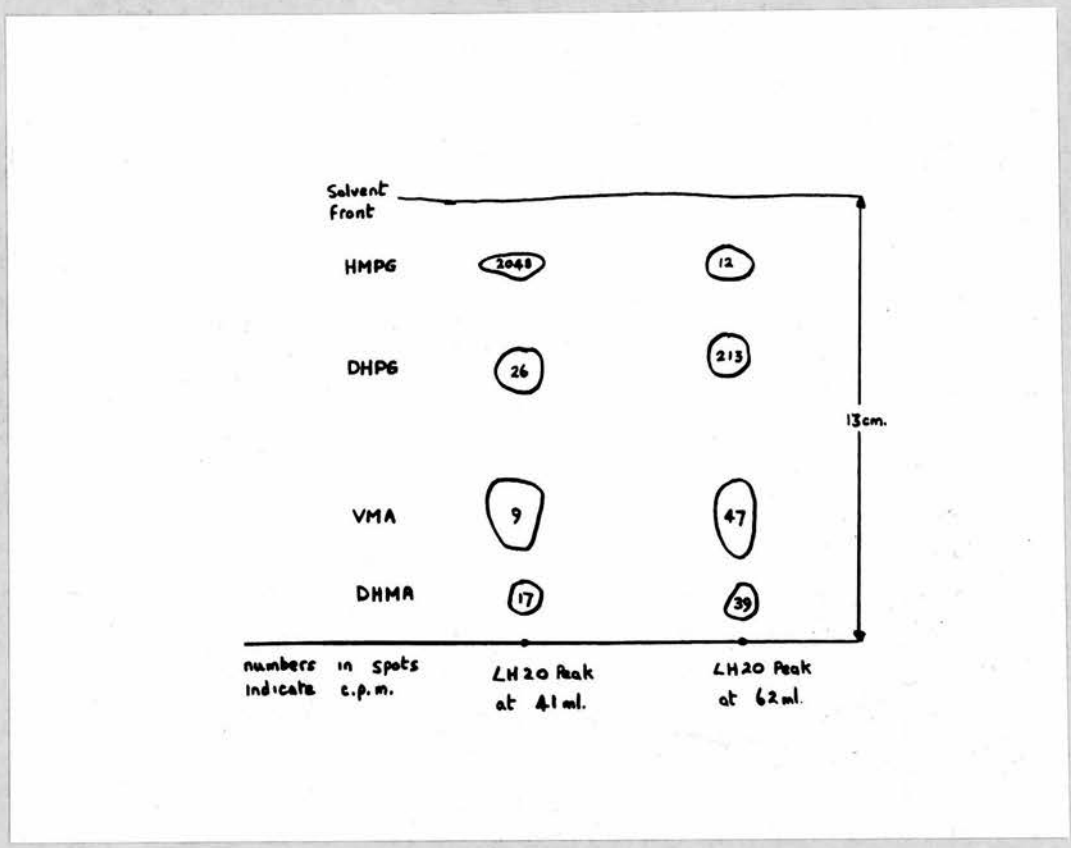

FIG.6 Thin layer chromatography on a 20 cm x 20 cm cellulose plate, of the concentrated, radioactive, LH20 column effluent samples at 41 ml and 62 ml, with marker amounts of HMPG, DHPG, VMA and DHMA, developed in chloroform: methanol: N ammonium hydroxide, 12:7:1.

Further indication that HMPG was contained in the LH 20 effluent peak at 41 ml was obtained by applying to the column 10 µg of HMPG contained in 0.2 ml of solvent mixture and monitoring the effluent using the fluorimetric method of Antun et al (1971), when a single peak of fluorescence was obtained at an effluent volume of 41 ml (Fig.5). Experiments were therefore designed to test the usefulness of the LH 20 column as a purification step in the extraction of HMPG from rat brain.

100 µg of HMPG was added to one rat brain, homogenised in 8 ml of distilled water, centrifuged and the supernatant extracted with 10 ml of petroleum ether, as before. The supernatant was buffered with the addition of $^{1}/_{10}$th volume of M sodium acetate buffer pH 5.0, and the glycol metabolites of NA extracted from the solution with 3x2 volumes of ethyl acetate. The extract was reduced to dryness in vacuo at 50°C, the residue resuspended in 0.2 ml of the LH 20 solvent mixture and applied to the LH 20 column. Effluent fractions monitored by fluorescence, showed a single peak after 41 ml consistent with previous results. The recovery through the method was 42%. When a similar experiment was attempted, but this time with 9 mg/ml of the crude enzyme preparation 'Helicase' added to the solution to hydrolyse the conjugated glycols with overnight incubation at 37°C, a totally different LH 20 effluent profile was seen. The crude enzyme preparation contained large amounts of fluorescent impurities which were nearly isographic with the peak of HMPG, with the result that no

purification of HMPG was possible in this system by the LH 20 column. Attempts to overcome this by altering the solvent characteristics and by purification of the 'Helicase' enzyme, were without success. The LH 20 column was, therefore, abandoned as a means of purification in experiments where it was necessary to have the sulphatase preparation present. Although the column was still useful for other experiments, the anion-exchange resin 'Bio-Rad' AG 1x4 was more convenient and equally as efficient, and was therefore included in the routine purification procedure for HMPG in preference to the LH 20 column.

2) <u>The use of 'Bio-Rad' AG 1 x 4 resin</u>

Antun et al (1971) described the use of the anion-exchange resin 'Bio-Rad' AG 1x4, 200-400 mesh in the chloride form, for the purification of urinary HMPG. The behaviour of the glycols on this resin was therefore investigated.

For convenience, columns were made from 5 ml safety pipettes with an internal diameter of 0.7 cm. A cotton wool plug was inserted at the base of the column, and 1.8 ml of a 1:1 suspension of the AG 1x4 resin in distilled water was added to form the resin bed.

A 1:1 suspension of the resin was prepared as follows: New resin was suspended in distilled water in a 200 ml beaker, allowed to settle over 15 min and the upper layer removed by decantation. This washing procedure was repeated a further two times, the resin made up once more

in distilled water and allowed to settle over 30 min. When a constant resin bed height was obtained, an equal height of water was retained over the resin, excess water being removed by suction through a pasteur pippette at the water pump. 1.8 ml of this suspension, thoroughly mixed just prior to use gave a consistent column resin bed height of 2.4 cm.

The behaviour of HMPG on the column was investigated by applying 0.5 ml of a 2 µg/ml solution of HMPG to the column, followed by successive 0.5 ml aliquots of distilled water. The eluate was collected for fluorescence analysis by the method of Antun et al (1971).

No HMPG was present in the sample effluent, or in the next 2.5 ml of eluate, but was totally eluted in the succeeding 4 ml (Figure 7).

The behaviour of DHPG on the column was investigated by monitoring the eluate by gas liquid chromatography (GLC). 0.5 ml of a 1 ug/ml solution of DHPG in distilled water was applied to the column, followed by successive 2 ml washes of distilled water. The 2 ml aliquots of eluant were made up to 4 ml with distilled water, and the solution acetylated, extracted, dried and trifluoracetylated, and the amount of DHPG present assessed by GLC as described later.

DHPG was retarded more by the AG 1x4 resin than HMPG. The dihydroxyphenyl glycol started to appear in the eluant at the same point as HMPG, but twice the volume was needed for its complete elution from the column (Fig.7).

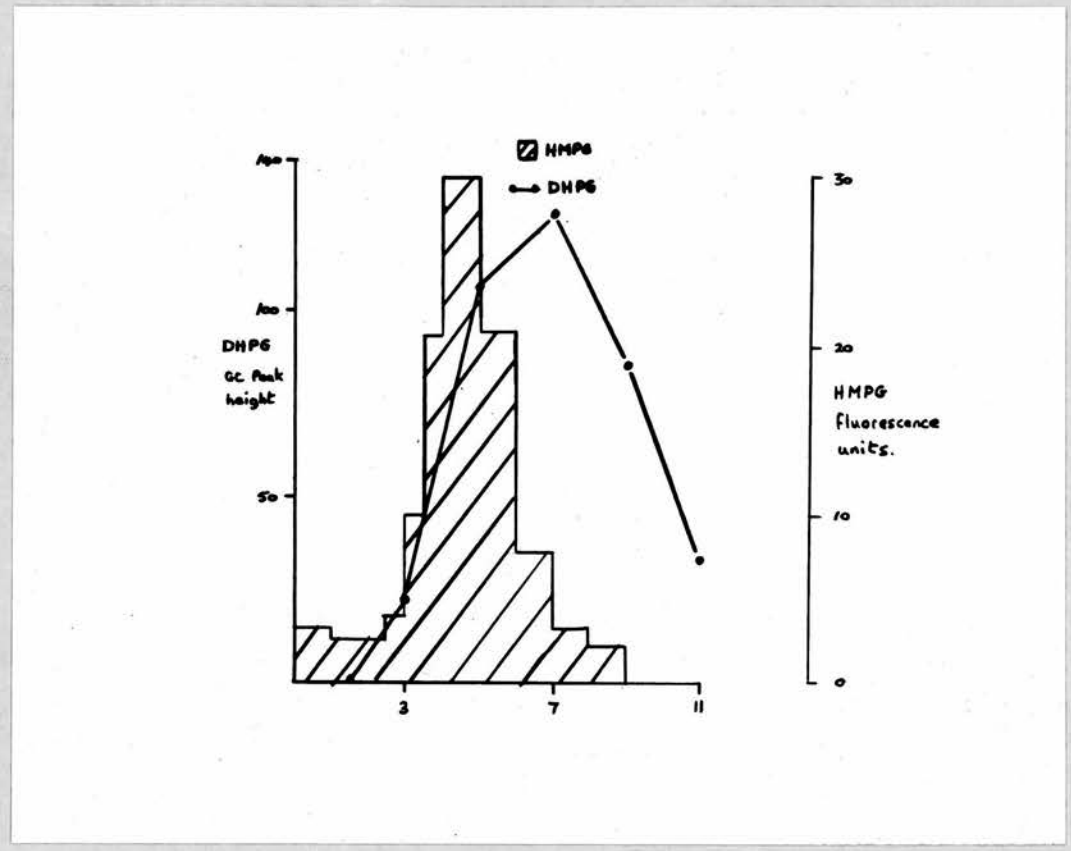

FIG.7 Chromatography, on a 2.7 cm x 0.7 cm column of 'Bio-Rad' AG1x4 resin, of HMPG (shaded histogram) and DHPG (solid line).

Although the glycols possess no full charge, their behaviour on this resin suggests that they are partially ionised, probably at the phenol group, since DHPG with the catechol group is retarded to a greater extent than HMPG.

Identification of the glycol metabolites using TLC

Fleming and Clark (1970) have described a two-dimensional thin layer chromatography system for the separation of the catecholamines and their metabolites. Their solvent system for the second direction is adequate to give a good separation of VMA, DHMA, HMPG and DHPG. This system was therefore employed for the further identification of the glycol metabolites of NA.

1 µl each of 1 mg/ml solutions of VMA, DHMA, DHPG and HMPG were applied to a 20 cm x 20 cm cellulose thin layer plate and the plate placed in solvent system 2 of Fleming and Clark 1970, which was a mixture of chloroform, methanol and N ammonium hydroxide in the proportions of 12:7:1. After two hours, when the solvent front had risen to 13 cm, the plate was removed, placed on a flat surface and dried with cold air, using a hair dryer. Visualization of the spots was made using a solution of diazotised p-nitroaniline, which substance will combine with phenol groups, and at alkaline pH, form coloured diazonium salts. The reagents were made up as follows.

1) 0.1 g p-nitroaniline was dissolved in 2 ml of concentrated HCl and diluted to 100 ml with distilled water.

2) 0.2 g of $Na\ NO_2$ in 100 ml of distilled water.

3) 20 g of $K_2\ CO_3$ in 200 ml of distilled water.

Diazotisation of the p-nitroaniline was achieved by mixing 1 volume of solution 1 with 1 volume of solution 2, in ice for 10 min. The dry plate was sprayed evenly with this mixture, allowed to dry, and then sprayed with the 10% potassium carbonate solution. Spots immediately appeared on the plate, which were either brown-red or purple in colour (Fig.6). When the plate was dry, the position of the spots was marked with a pencil in case of fading.

When radioactively labelled metabolites were chromatographed with this system, spots were scraped from the dry plate and the powder placed in scintillation vials to which was added 6.5 ml of a toluene based scintillant containing 0.425% w/v PPO and 0.0112% w/v POPOP.

A blank was obtained by scraping from the plate an area of cellulose equivalent in size to that of the metabolite spot from a position on the plate through which the solvent mixture had run.

Separation of the glycol conjugates by paper chromatography

Separation of the conjugated glycol metabolites of NA was made using the paper chromatography method described by Sugden and Eccleston (1971).

1 ml aliquots of the aqueous solution, containing the radioactively labelled conjugates, which remained after extraction of the amines, acid metabolites and the free

glycols (described earlier), were applied under a stream of nitrogen to 4 cm wide strips of 'Whatman' No 1 paper (Fig.8) and developed by descending chromatography, in solvent saturated tanks using a solventmixture of chloroform, methanol and 0.88 w/v ammonia in the proportions of 12:7:1, with a development time of 20 hr. When dry, the developed chromatograms were marked off into 1 cm sections. The radioactivity on the two end chromatograms was established by cutting these papers into 1 cm strips and immersing the strips in a toluene based scintillant containing 0.425% w/v PPO and 0.0112% w/v POPOP. The radioactive profile of the end chromatograms was then matched with the remaining chromatograms, from which the appropriate areas containing the radioactively labelled conjugated glycols were cut, and the metabolites extracted from the paper using methanol.

Two areas of radioactivity were present on the chromatogram, onepeak between 11 cm and 20 cm which corresponded to conjugated DHPG, and another peak between 30 cm and 39 cm corresponding to conjugated HMPG (Fig.8) The total radioactivity extracted from the remaining chromatograms amounted to between 70,000 and 72,000 counts per minute per ml for the two conjugated fractions respectively, an approximate ratio of 1:1. These purified fractions of conjugated HMPG and DHPG were used to investigate the optimum conditions for their hydrolysis.

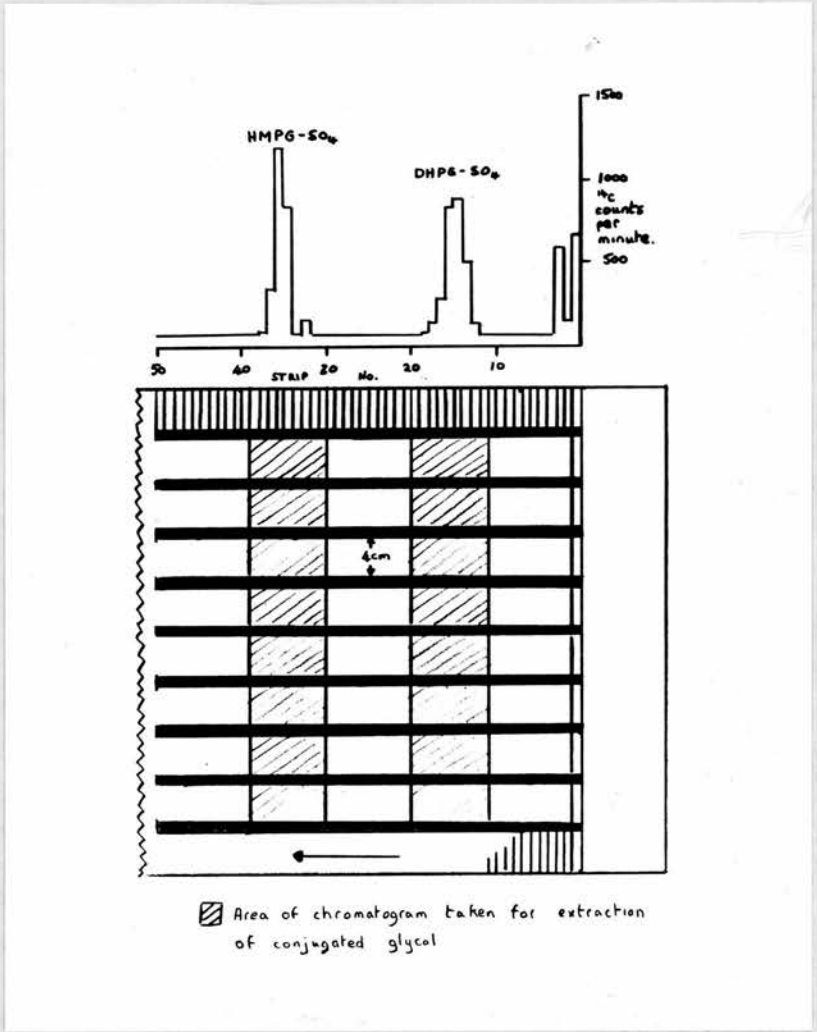

FIG.8 Upper. Chromatography of the aqueous solution containing (^{14}C)-labelled glycol conjugates remaining after the extraction of amines, acids and free glycol metabolites, following an intraventricular injection of (^{14}C) DL noradrenaline into rat brain.

Lower. Diagram of the paper chromatogram used to separate the glycol conjugates, matched with the radioactive profile, showing the areas from which the purified glycol conjugates were extracted. The arrow denotes the direction of development in chloroform, methanol, 0.88 ammonia, 12:7:1.

Hydrolysis of the conjugated metabolites of NA

In the absence of a method by which the conjugated metabolites could be analysed per se, it was decided, consistent with previous workers, to employ an enzymic hydrolysis of the conjugated metabolites, followed by the quantitative estimation of the liberated glycols.

The enzyme preparation used by most workers is a crude extract derived from the snail, Helix pomatia, known commercially as 'Helicase' or 'Glusolase', and contains known activity of sulphatase (15,000,000 Roy units per g for 'Helicase') and β-glucuronidase (1,000,000 Fishmann units per g) (1 Roy unit hydrolyses 1 µmole of p-nitrocatechol sulphate per hour at pH 5.0 and 37°C;).

Attempts were made to determine the hydrolysis rate curves using various concentrations of 'Helicase' in extracts from rat brain.

Aliquots of (^{3}H)-HMPG conjugate or (^{14}C) DHPG conjugate, prepared by the paper chromatographic method described earlier, were added to the aqueous solution which remained after the extraction of the other NA metabolites. The solution was buffered to pH 5.0 with the addition of one tenth volume of M sodium acetate buffer at pH 5.0, the 'Helicase' solution added, followed by two drops of chloroform, as an antibacterial agent. The tubes were then stoppered and incubated in a water bath at 37°C. The rate of hydrolysis of the conjugated glycols was determined from the amount of radioactivity which was

extracted from the incubate with ethyl acetate at pH 5.0.

The concentration of 'Helicase' generally used for urine hydrolysis (9 mg/ml) gave maximum hydrolysis of (^{3}H)-HMPG conjugate after 3 hours, whereas a tenth of this concentration (0.9 mg/ml) gave the same maximum after 17 hours incubation (Fig.9). A reduction in the amount of 'Helicase' added was therefore made, since a lowering of the amount of contamination was preferable to a shorter incubation time. To allow for any batch variations in the quality of the 'Helicase' preparation, however, a concentration of 2 mg/ml was chosen for subsequent work. This concentration of 'Helicase' was tested at a later stage measuring the rate of hydrolysis of endogenous (unlabelled) HMPG-conjugate by the rate of evolution of the free glycol, the latter measured by gas liquid chromatography (GLC) as described later.

The rate of hydrolysis of purified (^{14}C)-DHPG conjugate added to the rat brain conjugate fraction was determined after incubation with 2 mg/ml of 'Helicase', the concentration chosen as optimum for the hydrolysis of (^{3}H)-HMPG conjugate. Maximum hydrolysis occurred after 8 hours (Fig.10) a time similar to that found for HMPG-conjugate at this concentration of enzyme (Fig.9).

Quantitative estimation of HMPG by fluorimetry

Antun et al (1971) described a method for the fluorimetric determination of HMPG in human urine, based on the development of a fluorphore of HMPG after the addition of

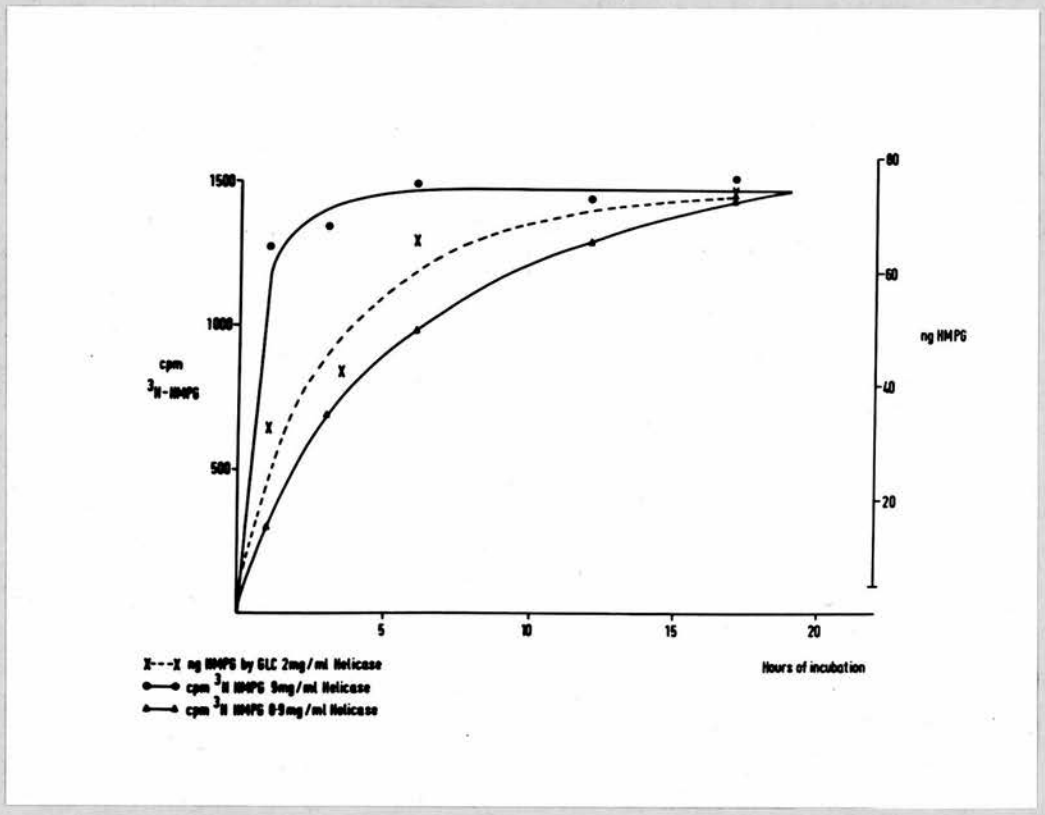

FIG.9 Rate curves showing the hydrolysis of HMPG-SO_4 at different concentrations of 'Helicase' enzyme preparations. Each point represents the mean of two determinations.

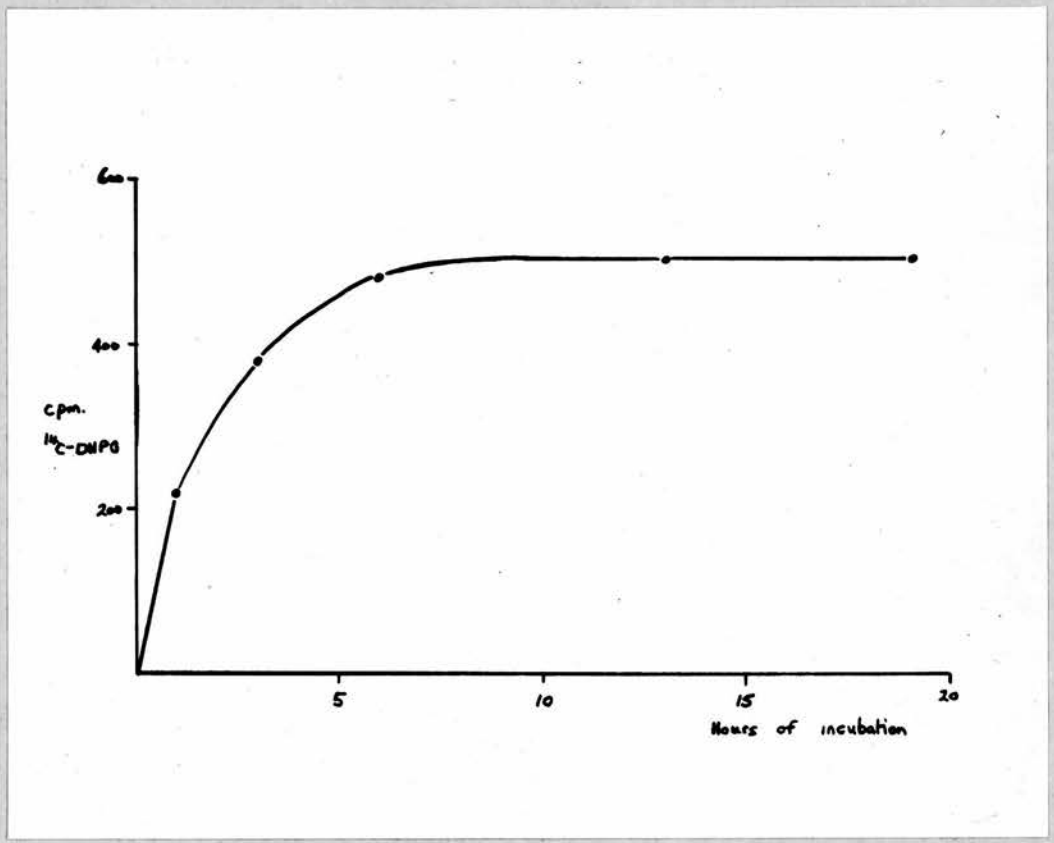

FIG.10 Rate curve showing the hydrolysis of (^{14}C)-DHPG-SO_4 using 'Helicase' enzyme preparation at a concentration of 2 mg/ml, showing that maximum hydrolysis occurred after about 8 hr.

ferric ions in a dilute solution of sodium hydroxide, and Pullar (1971) has modified this method for the measurement of small amounts of the metabolite in human cerebro-spinal fluid (CSF). The applicability of the method to the measurement of HMPG in rat brain was therefore investigated.

0.5 ml of an aqueous solution containing HMPG was placed in a 1.5 ml disposable plastic tube with attached stopper ('Eppendorf' system), and 0.05 ml of a 1% w/v ferric chloride solution in 0.04M NaOH, freshly prepared, added to the HMPG solution with mixing. The fluorphore was allowed to form for exactly 2 min in the absence of UV-light, after which the reaction was stopped with the addition of 0.05 ml of a freshly prepared solution of 5N sodium hydroxide. The latter caused the precipitation of ferric ions as ferric hydroxide and this was removed by high speed centrifugation of the suspension at 12,000 g for 2 min in a micro centrifuge ('Eppendorf' system).

The fluorescence of the supernatant was determined using a 'Perkin Elmer' PE 450 automatic fluorimeter with the excitation and emission slit widths set at 6 mm and 12 mm respectively. The emission wavelength was set at 415 nm and an excitation wavelength scan made from 230 nm to 380 nm. Fig.11 shows the fluorescence trace obtained with a) HMPG in distilled water and b) distilled water.

The linearity and sensitivity of the method in pure solution was determined using serial dilutions of an aqueous solution containing 820 ng/ml of HMPG, the results of which are shown in Fig.12. There was a linear

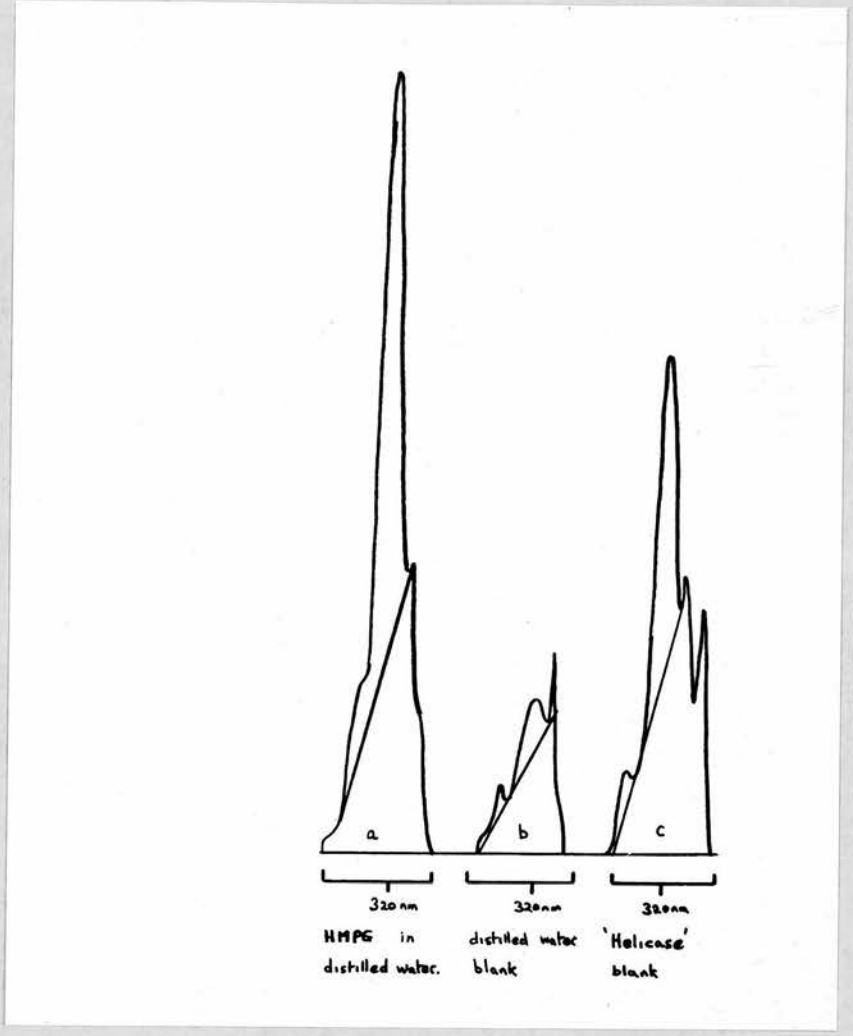

FIG.11 Excitation wavelength scans from 230 nm to 380 nm, with the emmission wavelength at 415 nm, the excitation slit width 6 mm and the emmission slit width 12 mm, of

a) the fluophore of an aqueous solution of HMPG, developed after 2 min treatment with ferric ions in dilute Na OH.

b) a distilled water blank under the same conditions.

c) a reagent blank taken through the method for the quantitative estimation of HMPG by fluorimetry including hydrolysis with 'Helicase'.

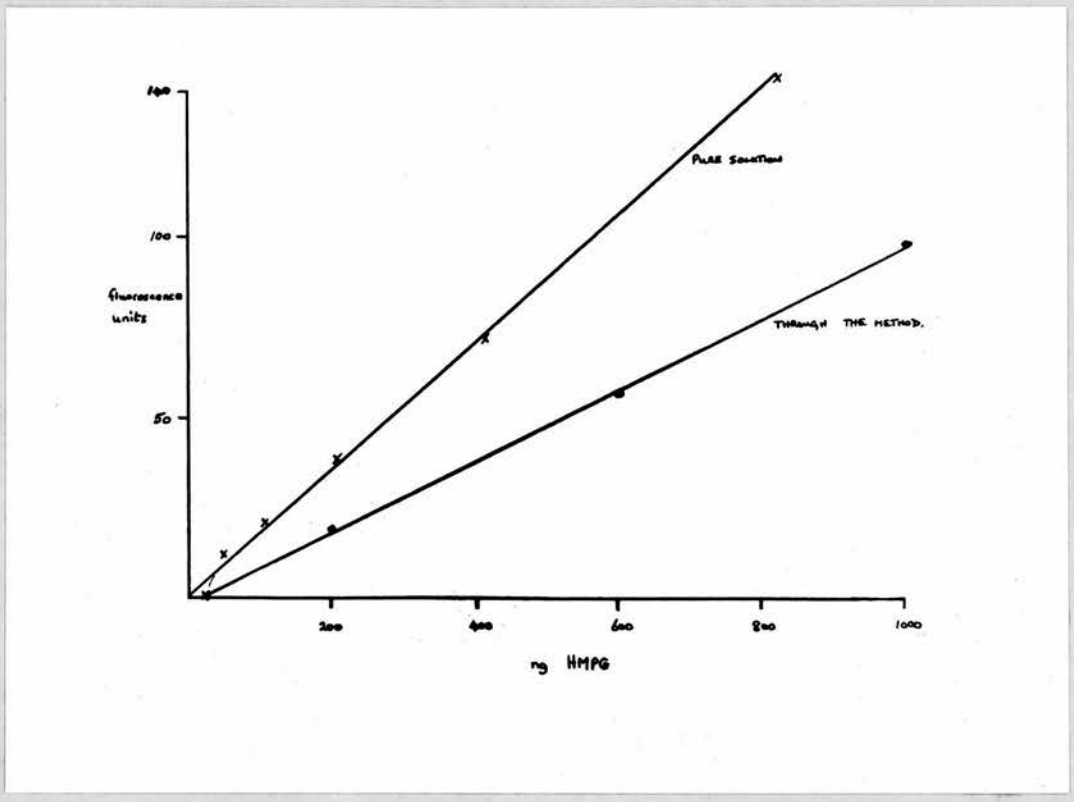

FIG.12 Graph showing a) the linear relation between the concentration of HMPG and the fluorescence peak height at 320 nm (minus the blank) produced after the reaction with ferric ions for 2 min.
and b) a similar linear relation for HMPG added to pooled rat brain and taken through the method of extraction and purification.

correlation between the concentration of HMPG in the sample (range 30 ng/ml to 820 ng/ml) and the fluorescence obtained. The sensitivity of the fluorimetric procedure taken as being the amount of HMPG giving a fluorescence intensity equal to twice the blank, was 30 ng in pure solution.

Since the formation of the fluorphore of HMPG in pure solution was found to have a linear relation with respect to the concentration of HMPG (Fig.12) the linearity of the extraction method was tested for increasing amounts of HMPG added to pooled samples of brain.

Four rat brains were each homogenised in 8 ml of hot distilled water in an all glass homogeniser. After pooling the homogenates, aliquots were taken and quantitatively transferred to test tubes containing standard amounts of HMPG, placed in a water bath at 100°C and incubated for 10 min. The flocculent precipitate of brain protein was removed by low speed centrifugation at 3000g for 15 min at 5°C and the supernatant transferred to a test tube containing 10 ml of petroleum ether (40°-60°C boiling range). The tubes were stoppered and shaken for 3 min to remove lipid, after which the tubes were centrifuged at 3000 g for 5 min to separate the layers. The petroleum ether (upper) layer containing extracted lipid was then removed by suction through a pasteur pipette at the water pump. The remaining lower layer was adjusted to pH 7.5 with KOH and the amines removed on a column of 'Amberlite' CG50 resin as described earlier. The combined column

effluent and wash was adjusted to pH 1.0 with concentrated HCl and the acid metabolites extracted by shaking with 3 x 2 volumes of ethyl acetate, discarding the extract. Residual traces of ethyl acetate were removed from the aqueous layer in vacuo at 50°C, the solution adjusted to pH 5.0 with KOH, and buffered at this pH with the addition of a tenth volume of M sodium acetate buffer pH 5.0. To each tube was added 10 mg of 'Helicase' enzyme preparation followed by 2 drops of chloroform as an antibacterial agent and the stoppered tubes incubated for 17 hours at 37°C. HMPG was extracted from the incubate at pH 5.0 by shaking with 3 x 2 volumes of ethyl acetate. This extract was reduced to dryness in vacuo at 50°C, the residue resuspended in 0.5 ml of distilled water, and this applied to a column of 'Bio Rad' AG1x4 resin as described earlier. After washing the column with 2.5 ml of distilled water, the HMPG was eluted in 4 ml of distilled water which was then reduced to dryness in vacuo at 50°C using a 'Edwards' high pressure vacuum pump. The residue was resuspended in 0.6 ml of distilled water, 0.5 ml of which was transferred to a 1.5 ml disposable plastic tube for the fluorimetric estimation of HMPG as described above. A reagent blank was taken through the method.

Fig.12 shows that the recovery of 200 ng, 600 ng and 1000 ng of HMPG added to pooled rat brain and taken through the method had a linear relation to the fluorescence of the samples, but with a reduction in sensitivity. This loss in sensitivity could be attributed to the increase in

the value of the method blank by a factor of six over that for the distilled water blank (Fig.11c) and to the limited recovery of HMPG through the method (25%). Antun et al (1971) described the halving of the sensitivity of the fluorescence method when applied to urine samples, giving a limit of sensitivity of 100 ng. There is a greater loss of sensitivity with the method described here for brain. Even though most of the impurity from the 'Helicase' preparation is removed during organic solvent extraction and chromatography with AG1x4 resin, there is sufficient residual contamination in the sample to limit the sensitivity of the method to about 200 ng where this amount of HMPG added to brain and taken through the method gives a fluorescence reading of twice the blank value.

Although this fluorescence method represents an accurate, fast and reproducible technique for the measurement of microgramme amounts of HMPG, either in pure solution or extracted from biological material, its applicability to the accurate estimation of the glycol in rat brain is limited, since the reported concentration of HMPG in brain is in the region of 40-60 ng/g (Schanberg et al 1968a; see also Results, this section). This technique was therefore reserved for the measurement of larger amounts of HMPG, and a more sensitive method sought for the quantitative analysis of the glycol in rat brain.

The quantitative estimation of HMPG and DHPG by gas-liquid chromatography (GLC).

The successful application of GLC methods to the measurement of HMPG in brain tissue has been made by two groups. Schanberg et al (1968a) reported values of free and total HMPG in rat, guinea pig, cat, rhesus monkey and green monkey brain, using the method of Wilk et al (1967) which employs the formation of a trifluoracetyl derivative of HMPG, whereas Sharman (1969) measured the concentration of free HMPG and DHPG in rabbit, mouse, and cat hypothalamus, following the formation of acetyl, heptafluorobutyryl derivatives of the glycols. Attempts were made therefore to apply these methods to the measurement of both free and conjugated HMPG in rat brain. The successful method for the estimation of HMPG was then modified at a later stage to incorporate the simultaneous measurement of DHPG.

For two reasons, it was decided to follow the method of Sharman (1969) rather than that of Wilk (1967), and form the acetyl heptafluorobutyryl derivative of HMPG. These were, firstly, the prior acetylation of the phenol group is thought to stabilize the later heptafluorobutyrylation of the alcohol groups and secondly, the formation of the heptafluorobutyryl derivative rather than the trifluoracetyl derivative should increase the electron capture detector (ECD) response, due to the greater number of fluorine atoms attached to the derivative.

The problem was approached in three methodological steps. These were

a) the formation and purification of a derivative of HMPG in bulk, to determine its chromatographic characteristics with the GLC system;

b) determination, in pure solution of the linearity of the derivative formation with respect to the ECD response;

c) determination of the linearity of the extraction procedure from brain, coupled with the derivative formation, with respect to the ECD response.

The following reagents and chemicals were used in the method:- HMPG; bis (4;hydroxy 3 methoxyphenyl glycol) piperazine salt from 'Sigma', sodium hydrogen carbonate, anhydrous sodium sulphate, hexachlorocyclohexane, acetic anhydride, ethyl acetate, 1:2, dichlormethane, trifluoracetic anhydride, phosphorous pentoxide and hexane, all from 'British Drug Houses Ltd' and 'Analar' grade, 1:2 dichlormethane puriss, heptafluorobutyric acid from 'Koch-Light' and ethyl acetate -CT from 'Reeve Angel'.

The use of electron capture detection in gas chromatography demands that all reagents be as pure as possible, to avoid confusion of the GLC trace with impurities. For this reason, all solvents were carefully redistilled before use. The two reagents contributing the most impurity to the final sample were ethyl acetate and 1:2 dichlormethane, large amounts of these being reduced to dryness during the method, thereby concentrating the impurities. Although these solvents were carefully distilled with a fractionation column prior to use, when 20 ml of either solvent was

reduced to dryness, the residue made up in 0.3 ml of ethyl acetate and 10 µl of the solution injected onto the column, the GLC trace obtained, still contained large peaks of impurity. This problem, however, was overcome by using a different source of solvents. 'Koch-Light' produce a 99.9% pure (GLC) form of 1:2 dichlormethane and 'Reeve Angel Scientific' produced a very pure (gas) Chromatographically Tested (CT) ethyl acetate - CT, both solvents giving clean GLC traces with the system described, without prior distillation.

a) <u>The formation and purification of electron-capturing derivatives of HMPG</u>

<u>Acetylation</u>

2 mg of HMPG-piperazine salt was dissolved in 4 ml of distilled water, to which was then added 0.3 ml of acetic anhydride followed by 0.6 g of sodium hydrogen carbonate. The mixture was gently shaken to promote the reaction which was allowed to complete over 30 min. Acetyl-HMPG was extracted from the aqueous solution by shaking for 3 min with 10 ml of 1:2 dichlormethane. Nine ml of the lower organic layer was removed, and transferred to a test tube containing 1 g of anhydrous sodium sulphate, stoppered and the mixture shaken. Residual traces of water were absorbed by the anhydrous sodium sulphate, which was removed from the dry extract by filtering the mixture through a micro Buchner funnel with a scintered glass disc, at the water pump. The dry extract was then reduced to dryness in

vacuo at 50°C. The clear viscous oil of acetyl-HMPG remaining after the evaporation of the dichlormethane was then redissolved in 2 ml of ethyl acetate and kept at -20°C in a stoppered test tube as starting material for the next stage of the derivative formation.

Heptafluorobutyrylation

Heptafluorobutyric anhydride (HFB anhydride) was prepared from the dehydration of heptafluorobutyric acid (HFB acid) with phosphorous pentoxide (P_2O_5). 100 g of HFB acid was carefully poured on to 100 g of P_2O_5 (Molar ratio of 1:1.5) in a 2-litre round bottom flask fitted with a reflux condenser. The mixture was heated under reflux for 3 hours after which the HFB anhydride was removed by distillation, collecting the fraction which boiled between 106°C and 110°C. The HFB anhydride was distilled a further time and then kept at room temperature in a stoppered 100 ml round bottom flask. Aliquots of the twice distilled HFB anhydride were taken just prior to use for further distillation, and the 3x distilled HFB anhydride used for the reaction with acetyl-HMPG.

0.1 ml of the purified HFB anhydride was added to 0.4 ml of the standard solution of acetyl-HMPG in ethyl acetate in a 2 ml borosilicate-glass reaction tube. The tube was closed with a plastic closure which made a tight fit round the outer wall of the tube. The reagents were mixed and placed in a micro-heating block ('Eppendorf' series) at 56°C for 15 min.

The plastic closure was then removed and the solution reduced to dryness under a stream of dry nitrogen (obtained by passing over a column of anhydrous calcium chloride) for a further 15 min whilst the tube remained in the heating block. When the reaction mixture had evaporated, the clear oil of acetyl, heptafluorobutyryl HMPG (acetyl HFB-HMPG) was redissolved in 1 ml of hexane, and transferred to a 15 ml conical centrifuge tube. Crystalline acetyl HFB-HMPG was obtained by cooling the hexane extract immersing the test tube in a freezing mixture of acetone and solid carbon dioxide. The heavy crystals sedimented sufficiently well to permit the rapid decantation of the supernatant. The crystals were dissolved in a further 0.5 ml of hexane and the procedure repeated.

The 2x crystalline derivative was finally resuspended in 1 ml of hexane and appropriate dilutions of this solution made for analysis.

Trifluoracetylation

It was found to be sufficient to redistil the trifluoracetic anhydride (TFA anhydride) obtained from 'BDH', once only, since the starting material was quite pure. About 10 ml of the fraction boiling between 39°C and 41°C was collected, by distillation under anhydrous conditions, in a 25 ml conical flask, and the stoppered flask kept at room temperature for up to 3 months without any deterioration in the efficiency of the reagent. The acetyl, trifluoracetyl derivative of HMPG (acetyl-TFA-HMPG) was prepared in a similar way to the acetyl-HFB-HMPG, after reaction of

0.4 ml of acetyl-HMPG with 0.1 ml of TFA anhydride, followed by 2x crystallization from hexane. This derivative, once formed is very stable, no deterioration in the ECD response being evident in a sample of the derivative in ethyl acetate, kept at room temperature for more than 12 months.

In each case, a reagent blank was processed alongside samples of HMPG to avoid confusion at the gas chromatography stage.

GLC of the derivatives of HMPG

Gas-liquid chromatography was performed using a 'Pye' 104 series gas chromatograph fitted with a (^{63}Ni)-electron capture detector. Fig.13 shows a schematic diagram of the gas chromatographic system.

Argon/methane carrier gas (95:5), flowing in $^{1}/_{8}$ inch (OD) copper piping at a pressure of 0.9 kg/cm^{3} was passed through a gas purifying bottle containing an activated molecular sieve, and connected directly to the head of the heated injection port, by-passing the gas flow controllers. (This was because previous difficulty with impurity was shown to originate from the gas flow controllers.) The flow rate, instead, was regulated directly using the gas cylinder pressure gauge, flow rate measured at the gas exit with a soap bubble flow meter.

The injection head was screwed, with a gas tight joint, to a 5 ft glass column $\frac{1}{4}$ inch outside diameter filled with a celite supportprecoated uniformly with 3% methyl silicone gum in methylene chloride ('Pye' 3% E30), the powder kept

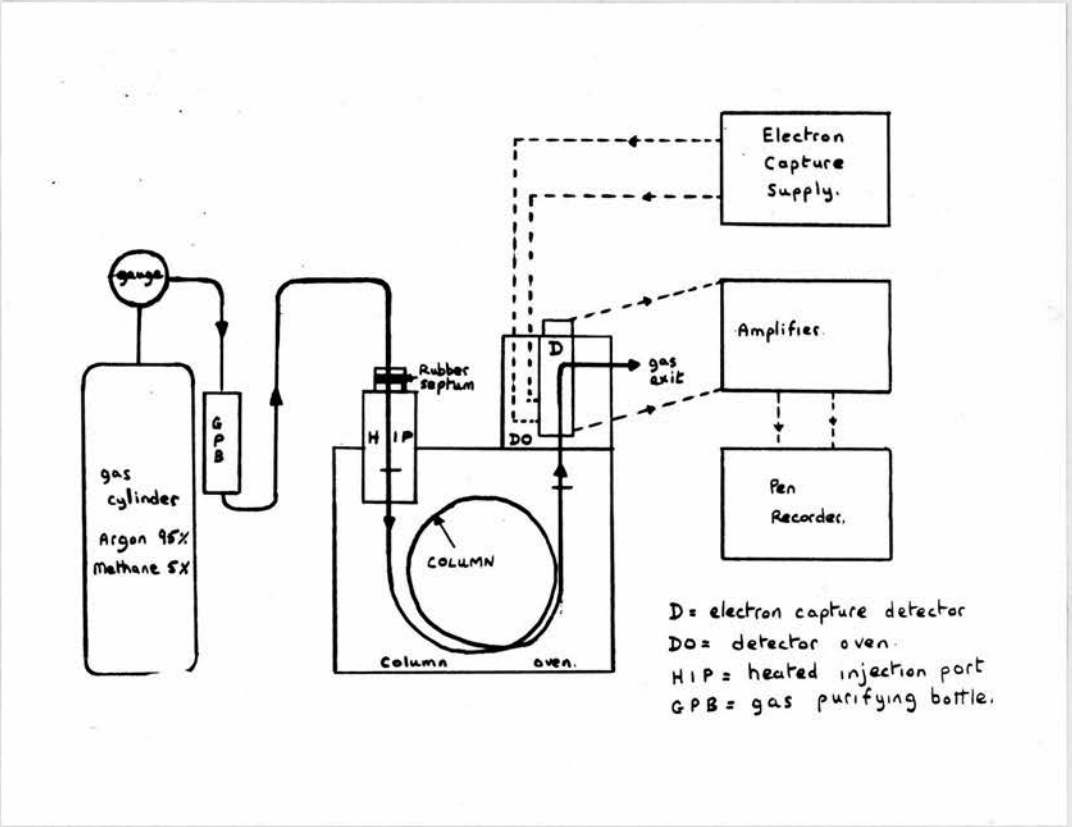

FIG.13 A schematic diagram showing the experimental system for gas chromatography. The column oven and detector oven power supply and temperature control units have been omitted for the sake of clarity.

in position with a yarn plug. Carrier gas left the column through a connection at the base of the electron capture detector through which it then flowed and found its exit. No purge gas was necessary in this system.

Samples were introduced into the system by on-column injection of between 1 µl and 5 µl of the ethyl acetate solution, using a 10 µl glass micro syringe with an 11.5 cm long needle. The needle was pushed quickly through the rubber septum at the injection port head, the sample injected, and the needle quickly withdrawn. After between 10 and 15 injections, the rubber septa (2) were replaced, removing the septum retaining screw for this purpose. It was unnecessary to first reduce the pressure of gas, a back-pressure of column packing material being prevented by the yarn plug.

Column oven temperature (190°C for HMPG and 170°C for HMPG + DHPG) and detector oven temperature (250°C) were controlled and maintained with their separate control units each connected to a platinum resistance thermometer mounted inside the oven.

Fig.14 shows a detailed section through the 'Pye' electron capture detector showing a cylindrical source of (^{63}Ni) at the periphery of a small chamber, through which carrier gas + sample passes, surrounding a centrally displayed probe. The steady state current across the chamber from the source to the probe is maintained by the electron capture supply unit using either a direct current or a pulsed voltage supply. A pulsed voltage supply was

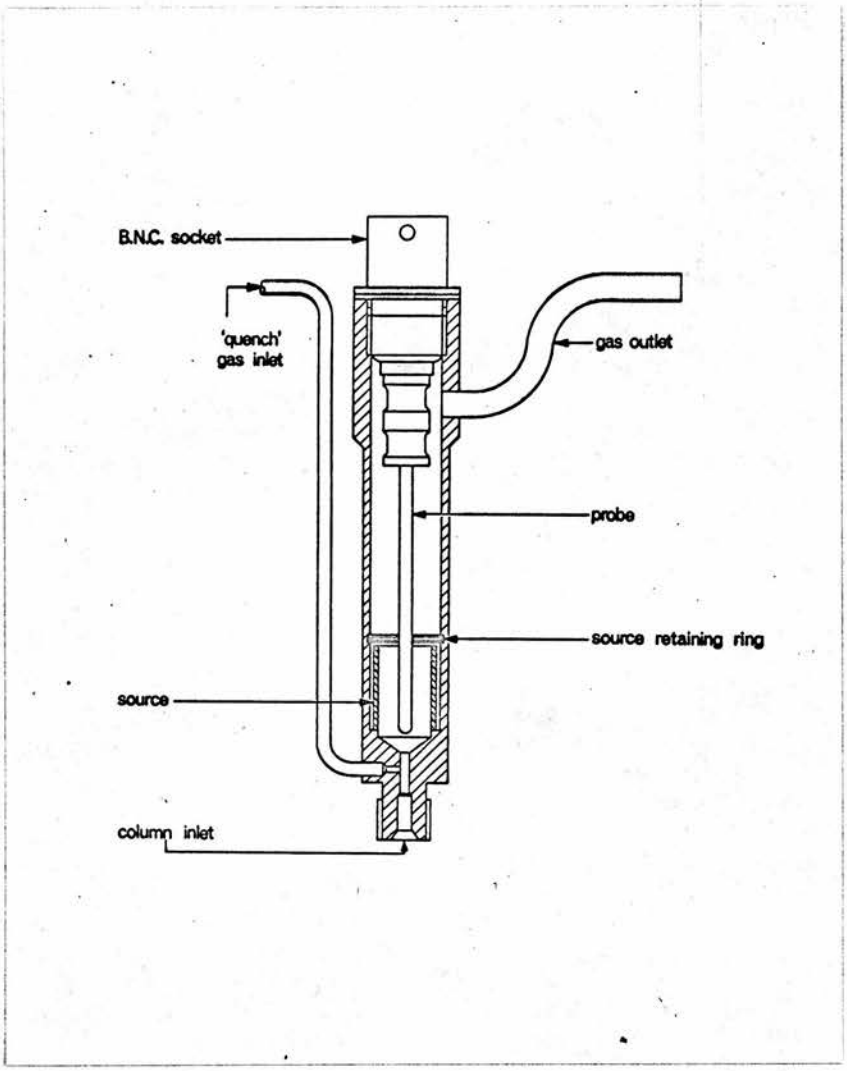

FIG.14 Diagram showing a detailed section through the 'Pye' electron capture detector.

employed here using a pulse period of 150 μs with a pulse width of 0.75 μs ± 0.25 μs, and having an amplitude of 47-60 volts.

Fig.15 shows the recorder trace from the injection of 2 μl of a dilute ethyl acetate solution of acetyl-HFB-HMPG, retention time 4.2 min, or acetyl-TFA-HMPG, retention time 3.4 min, using the gas chromatographic system described, with the column oven temperature at 190°C. The faster moving peak, retention time 5.4 min, in each case, is the marker substance hexachlorocyclohexane. 20 ng/ml of this compound in ethyl acetate gave approximately a half full-scale deflection at the attenuation normally used when estimating HMPG from rat brain. Samples were therefore made up in this standard solution, and could then be corrected for any variation in volume injected.

b) <u>Determination, in pure solution, of the linearity of the derivative formation</u>

Dilutions of a 2 μg/ml solution of HMPG were made to give the following amounts of the glycol in 4 ml of distilled water, 10 ng, 20 ng, 40 ng, 100 ng, and 150 ng, with a reagent blank. The aqueous solution was acetylated, then heptafluorobutyrylated or trifluoracetylated as described. After blowing to dryness in the heating block, using a stream of dry nitrogen, the tubes were removed from the block and the residue resuspended in 0.3 ml of a 20 ng/ml solution of hexachlorocyclohexane (HCH) in ethyl acetate. The formation of the acetyl HFB-HMPG and acetyl-

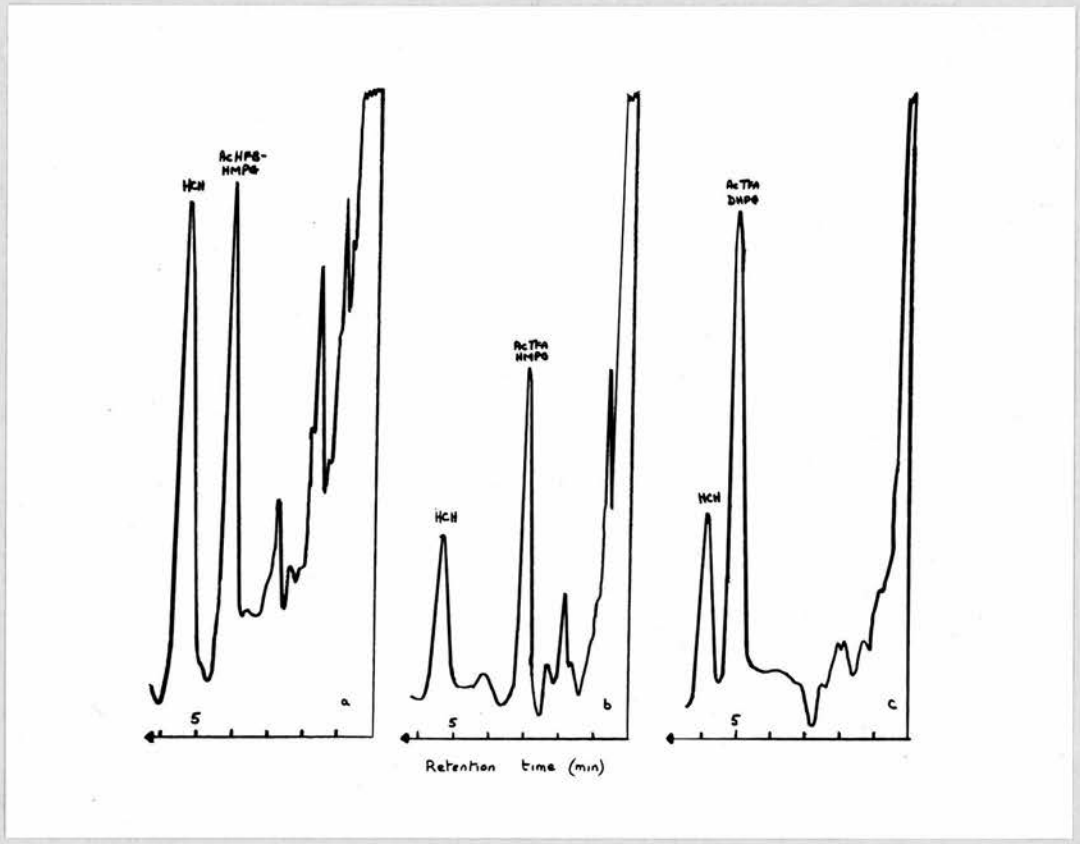

FIG.15 GLC recorder traces showing the retention of various glycol derivatives with respect to that of hexachlorocyclohexane (HCH).

a) Acetyl-HFB-HMPG (retention time 4.2 min) with HCH (5.4 min).

b) Acetyl-TFA-HMPG (3.4 min) with HCH (5.4 min).

c) Acetyl-TFA-DHPG (5.0 min) with HCH (5.9 min) Column oven temperature at 190° C.

TFA-HMPG derivatives was monitored by GLC. Fig.16 shows the linear relation between the starting concentration of HMPG and the ECD response for acetyl TFA-HMPG, when the peak height of the latter was adjusted with respect to the peak height of HCH to correct for the volume of injection. Table 3 shows the derivation of the H30 value, which is the acetyl-TFA-HMPG peak height adjusted with respect to a HCH peak height of 30 divisions.

The acetyl HFB derivative of HMPG is formed at all concentrations of HMPG used, but with a non-linear relation to the H30 value. So great was the variability on some occasions that double the concentration of HMPG used, gave a similar peak height. All attempts to remedy this, by altering the temperature and/or the time of the heptafluorobutyrylation reaction, or by varying the temperature and/or time for the removal of the reaction mixture, failed. For this reason, therefore, this derivative was not used in subsequent work.

c) <u>Determination of the linearity of the extraction procedure coupled with the derivative formation with respect to the ECD response</u>

Dilutions of a 2 µg/ml solution of HMPG were made to give samples containing from zero to 200 ng of the glycol which were added to aliquots of pooled samples of the perchloric acid homogenate of rat brain tissue, and taken through the method for the extraction and derivative formation of HMPG as summarized below.

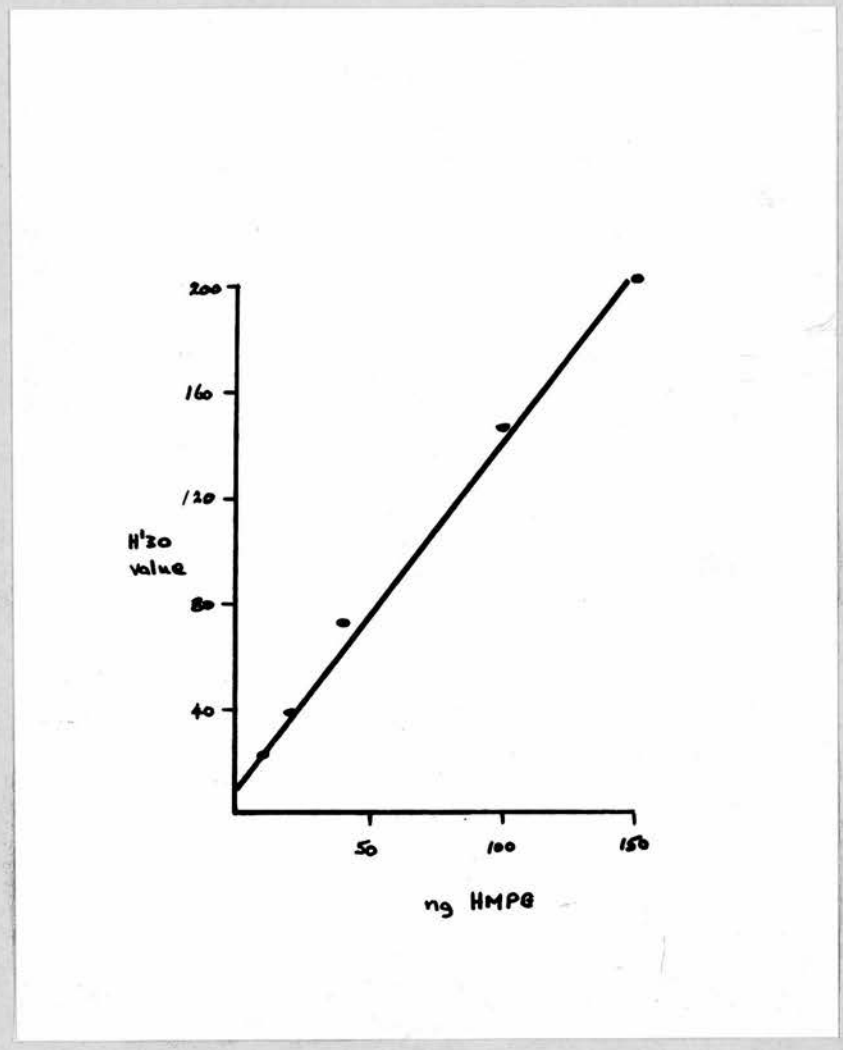

FIG.16 Graph showing the linear relation between the starting concentration of HMPG and the formation of the acetyl TFA derivative of HMPG, as measured by the GLC peak height of the derivative after adjustment for injection volume(H'30 value). Each point is the mean of two determinations. The best straight line was calculated from the original data by the method of least squares. r (correlation coefficient) = 0.9979

TABLE 3 Derivation of the H'30 value, obtained from the GLC peak heights of the acetyl-TFA-HMPG derivative and the internal standard of HCH.

$$\text{Adjusted acetyl-TFA-HMPG peak height (H'30 value)} = \text{Actual acetyl TFA-HMPG peak height} \times \frac{\text{HCH standard peak height of 30 divisions}}{\text{Actual HCH peak height}}$$

Weighed samples of brain tissue were homogenised in 5 ml of 0.4 M perchloric acid at 5°C with an all glass homogeniser, the homogeniser washed with a further 1 ml of 0.4M perchloric acid, the homogenate and wash combined and centrifuged at 10,000 g for 15 min at 5°C. The clear supernatant was adjusted to between pH 4.0 and pH 5.0 with 5 N and 0.1 N KOH by glass electrode. To obtain maximum precipitation of potassium perchlorate, the solution was cooled to 5°C for 15 min prior to low speed centrifugation at 3000 g for 10 min at 5°C. The clear supernatant was buffered to pH 5.0 with the addition of 0.5 ml of M sodium acetate buffer at pH 5.0, and 0.2 ml of a 50 mg/ml solution of 'Helicase' enzyme preparation (10 mg) added to each tube, followed by two drops of $CHCl_3$ as an antibacterial agent. The tubes were then stoppered, and placed in a water bath at 37°C for 17 hours (overnight). Following enzymic hydrolysis, HMPG (free) was extracted from the solution by shaking twice for 3 min with 15 ml and 10 ml of ethyl acetate. 12 ml and 10 ml of the extract in each case was removed, placed in a 100 ml round bottom flask and evaporated to dryness, in vacuo, at 50°C. The residue was resuspended in 0.5 ml of distilled water, and applied to a 2.4 cm x 0.7 cm column of 'Bio-Rad' AG1x4 resin (200-400 mesh in the chloride form). The flask and column were washed with 2.5 ml of distilled water and the HMPG eluted with a further 4 ml of distilled water.

The eluate was acetylated by the addition of 0.3 ml of acetic anhydride and 0.6 g of sodium hydrogen carbonate,

shaking gently to promote the reaction, and allowing 30 min for completion. Acetyl-HMPG was extracted from the aqueous solution by shaking for 3 min with 10 ml of 1:2 dichlormethane. Nine ml of the lower organic layer was removed, transferred to a test-tube containing 1 g of anhydrous sodium sulphate, stoppered, and shaken to remove residual traces of water. The dry extract was filtered through a micro-Buchner funnel with a scintered glass disc, and reduced to dryness, in vacuo, at 50°C. The residue was resuspended in 0.5 ml of ethyl acetate, 0.4 ml of which was transferred to a 2 ml borosilicate glass reaction tube.

0.1 ml of trifluoracetic anhydride was added to the tube, stoppered and placed in a micro-heating block at 56°C for 15 min after which the solution was blown to dryness under a stream of dry nitrogen whilst still in the heating block, for a further 15 min. The residue was resuspended in 0.3 ml of a 20 ng/ml ethyl acetate solution of hexachlorocyclohexane, and the sample analysed by injecting between 1 µl and 5 µl of this solution onto the gas chromatography column as described earlier.

Fig.17 shows the linear relation between the starting concentration of HMPG and the H30 value. The point of intersection of the line with the ordinate gives the endogenous concentration of total (free + conjugated) HMPG in the aliquot of pooled rat brain homogenate used. This method was used therefore for all subsequent determinations of HMPG in rat brain tissue. The quantitative

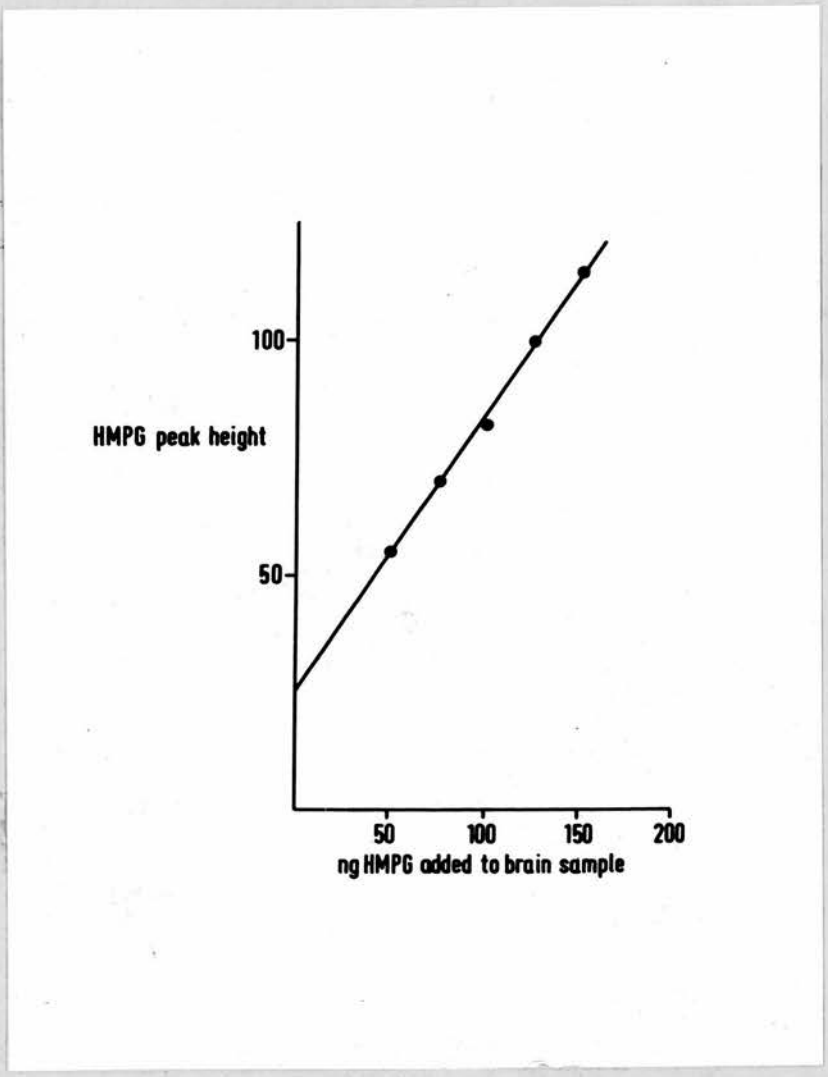

FIG.17 The recovery of HMPG added to pooled rat brain and taken through the method for the extraction, purification and acetyl-TFA derivative formation. The graph shows the linear relation between the starting concentration and the H'30 value. Each point is the mean of two determinations. The best straight line was calculated from the original data by the method of least squares.
r (correlation coefficient) = 0.9926
The point of intersection with the ordinate is the H'30 value for the endogenous concentration of HMPG in the pooled brain sample.

estimation of HMPG was made using an internal standard of 100 ng of HMPG added routinely to one of a pair of pooled brain tissue samples and taken through the extraction procedures and derivative formation. The difference between the sample and the sample + 100 ng HMPG gave the H'30 value equivalent to 100 ng of HMPG. This figure could then be used to calculate the concentration of HMPG in an unknown sample (table 4).

When it was important to measure the separate concentrations of free and conjugate HMPG, free HMPG was extracted before hydrolysis of the conjugated glycol, by shaking twice for 3 min with 15 ml and 10 ml of ethyl acetate as described above. Residual traces of ethyl acetate were removed, in vacuo, at 50°C, and a further 0.5 ml of M sodium acetate buffer pH 5.0 added to the aqueous solution before the addition of the 'Helicase' enzyme preparation. The free glycol was purified and the acetyl TFA-derivative made as described above.

The simultaneous quantitative estimation of DHPG and HMPG by GLC.

Since it is possible to form an acetyl-trifluoracetyl derivative of HMPG in a reproducible and quantitative manner, the formation of a similar derivative of DHPG was attempted. Similar methodological steps were taken to ensure that the derivative was being formed with a linear relation to the starting concentration of the glycol. Some modification of the extraction and purification

TABLE 4 Table showing the internal recovery method for calculating the concentration of glycol in a known weight of tissue.

H'30 value of pooled sample + 100 ng HMPG = x

H'30 value of pooled sample = y

$\therefore$ H'30 value of 100 ng HMPG = (x - y)

H'30 value of unknown = z

$\therefore$ ng of HMPG in unknown = $\frac{z}{(x - y)} \times 100$

so, concentration of HMPG in unknown = $\frac{z}{(x - y)} \times \frac{100}{\text{weight of tissue}}$

procedure for HMPG was then made, to permit the simultaneous extraction and purification of DHPG, and the linearity of the method tested by the relation between increasing amounts of DHPG added to pooled brain samples, and the ECD response of the formed derivatives.

a) Formation of an acetyl-trifluoracetyl derivative of DHPG

2 mg of DHPG was dissolved in 4 ml of distilled water and acetyl-DHPG formed as described for HMPG, by the addition of 0.3 ml acetic anhydride and 0.6 g of sodium hydrogen carbonate. An aliquot of acetyl-DHPG was then trifluoracetylated as described for HMPG and the acetyl TFA-DHPG purified by 2X crystallisation from hexane. Fig.15c shows the recorder trace of a 2 µl injection of a dilution of the acetyl-TFA-DHPG in a 20 ng/ml ethyl acetate solution of hexachloracyclohexane. Acetyl TFA-DHPG is a slower moving derivative than acetyl TFA-HMPG in this system, having a retention time of 5.0 min when the retention time of HCH was 5.9 min.

b) Determination of the linearity of the derivative formation

Zero, 50 ng, 100 ng, 150 ng, 200 ng and 250 ng amounts of DHPG were acetylated, then trifluoracetylated, the derivative made up in 0.3 ml of an ethyl acetate solution containing 20 ng/ml HCH, and 2 µl of the sample injected onto the gas chromatography column for analysis. Fig.18 shows that there was a linear relation between the starting concentrations of DHPG and the acetyl-TFA-DHPG peak height, adjusted with respect to the HCH peak height, thus

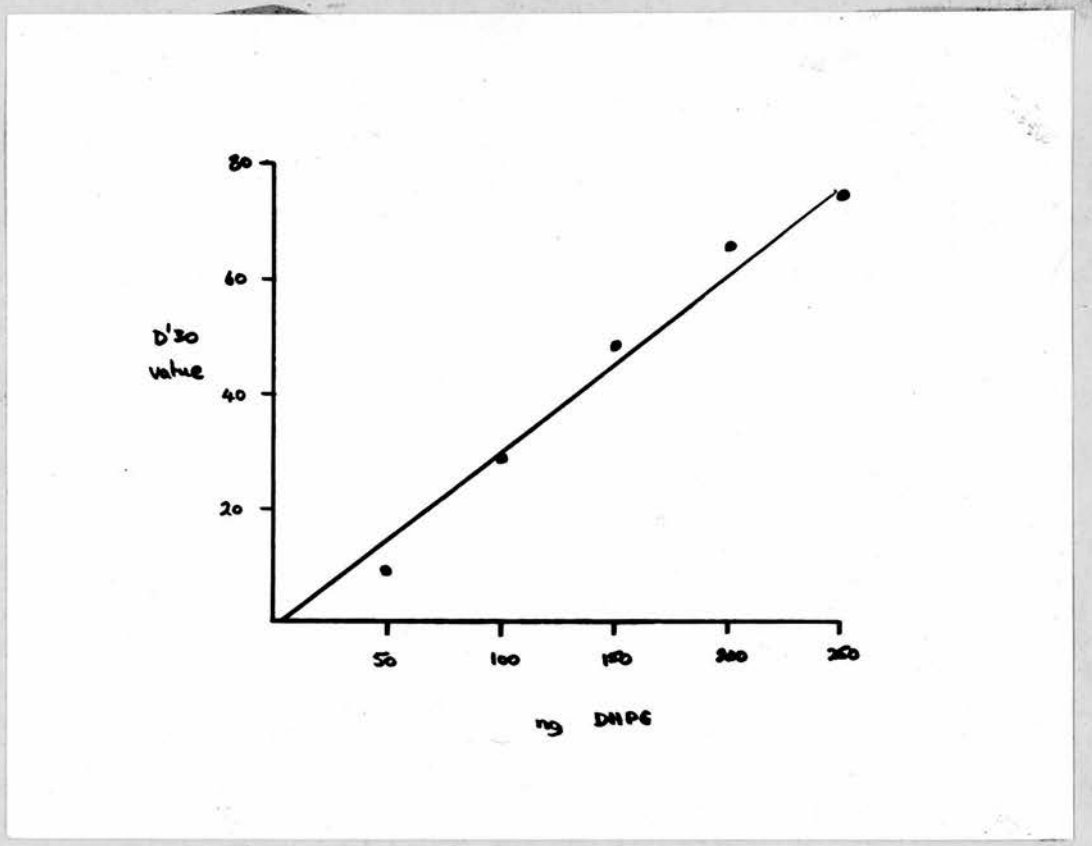

FIG.18 Graph showing the linear relation between the starting concentration of DHPG and the formation of the acetyl-TFA derivative of DHPG as measured by the GLC peak height of the derivative after adjustment for injection volume (D'30 value). Each point is the mean of two determinations The best straight line was calculated from the original data by the method of least squares. r (correlation coefficient) = 0.9898

correcting for variation in injection volume. (D'30 value, calculated as for HMPG in table 3.)

c) Modification of the method for the quantitative estimation of HMPG from rat brain, to permit the simultaneous quantitative estimation of DHPG.

DHPG was found to behave identically with HMPG in the extraction procedure up to the stage of purification of the glycols using a column of 'Bio Rad' AG1x4 resin. The behaviour of DHPG on this resin is described earlier, and summarised in Fig.7. DHPG was retarded more than HMPG by the resin and although the glycol started to appear in the eluate at the same point as HMPG, twice the volume of distilled water (8 ml) was needed for its complete elution from this column.

Because there was double the volume of water at this stage, double the amounts of acetic anhydride and sodium hydrogen carbonate were used in the subsequent acetylation reaction, and a larger volume of 1:2 dichlormethane used to extract the acetylated glycols.

The revised method was as follows:- ethyl acetate extracts containing the glycols were reduced to dryness in the usual way, and the residue resuspended in 0.5 ml of distilled water. This was applied to a 2.4 cm x 0.7 cm column of 'Bio-Rad' AG1x4 resin (200-400 mesh in the chloride form), the flask and column washed with 2.5 ml of distilled water, and the DHPG and HMPG eluted with a further 8 ml of distilled water.

0.6 ml of acetic anhydride and 1.2 g of sodium hydrogen carbonate were added to the 8 ml of column eluate. The mixture was gently shaken to promote the reaction and allowed to complete over 30 min. Acetyl-HMPG and acetyl-DHPG were extracted from the aqueous solution by shaking for 3 min with 15 ml of 1:2 dichlormethane. 12 ml of the lower organic layer was removed and transferred to a test tube containing 1 g of anhydrous sodium sulphate, stoppered and shaken to remove residual traces of water. The extract was then filtered, reduced to dryness, resuspended and trifluoracetylated as previously described.

The conditions for GLC had to be slightly altered to permit the simultaneous estimation of HMPG and DHPG, since with a column temperature of 190°C used in the estimation of HMPG alone, the acetyl TFA-DHPG peak was isographic with HCH. A column temperature of 180°C was similarly useless since at this temperature, acetyl TFA-HMPG was isographic with a large contaminant peak. When a column temperature of 170°C was used it was found that both acetyl TFA-HMPG and HCH were uncontaminated, but the acetyl-TFA-DHPG was nearly isographic (within 0.4 min) with a small contaminant peak. Because blank determinations showed that this small peak had a constant low value, it was decided to use a column temperature of 170°C and subtract from the DHPG derivative peak height the value of the contaminant peak height at the acetyl-TFA-DHPG position.

The lowering of the column oven temperature by 20°C caused a slowing of the retention times of the derivatives. This was overcome by increasing the flow rate of the carrier gas, adjusting the gas pressure to 1 Kg/cm^3.

Fig.19 shows a typical recorder trace of a sample derived from rat brain, and a superimposed method blank (the latter shown as a dotted line in the region of the acetyl-TFA-DHPG peak and the HCH peak position). The retention times with this system were, acetyl-TFA-HMPG, 4.9 min; acetyl TFA-DHPG, 7.8 min; and HCH, 8.6 min.

Fig.20 shows the recovery of increasing amounts of both HMPG and DHPG added to the same aliquot of pooled brain tissue and taken through the modified method. Both HMPG and DHPG show a linear relation between the starting amounts of the glycols and the adjusted peak heights of the derivatives, calculated from the same sample. The points of intersection of the two curves with the ordinate give the peak heights of the endogenous concentration of the glycols in the pooled sample of brain. **The slopes of the lines, calculated by the correlation of linear regression, are almost parallel (HMPG slope = 0.241; DHPG slope = 0.212) indicating that the recovery of the two glycols through the method is nearly the same.**

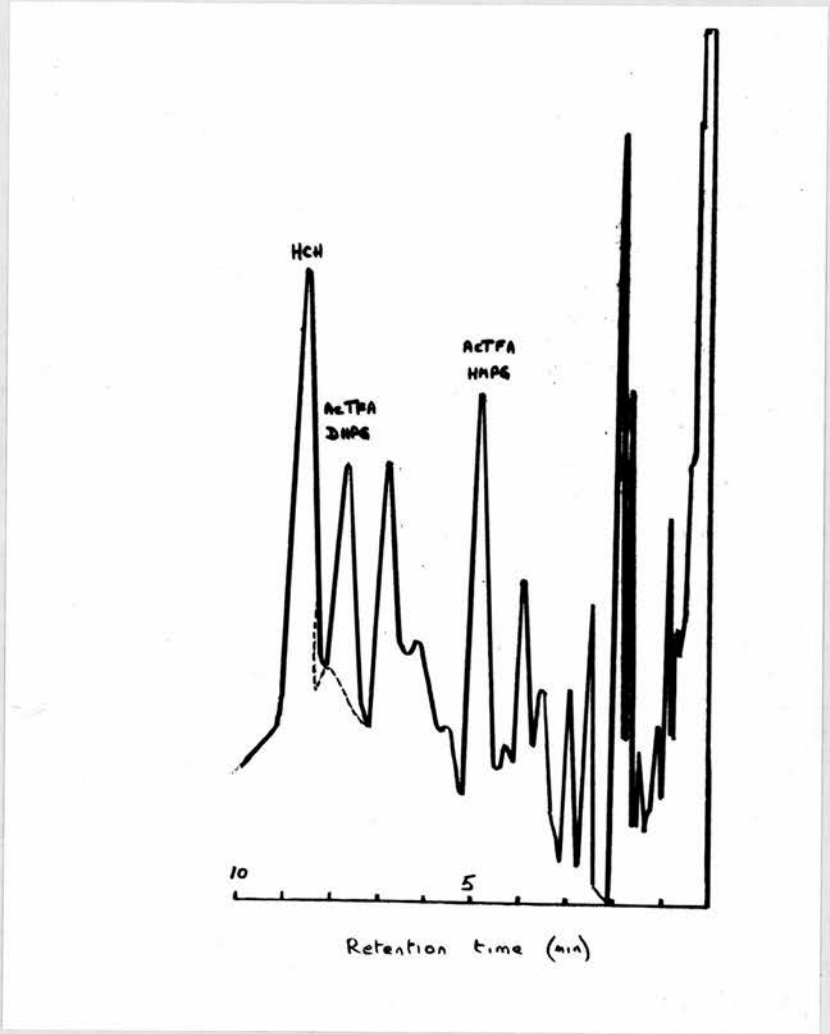

FIG.19 G.C. recorder trace of a sample from rat brain showing retention times of acetyl-TFA-HMPG (4.9 min), acetyl-TFA-DHPG (7.8 min) and HCH (8.7 min). The dotted line indicates the appearance of the blank at this position. Column oven temperature 170°C, ECD temperature 250°C, argon: methane carrier gas 95:5 at 40 ml/min.

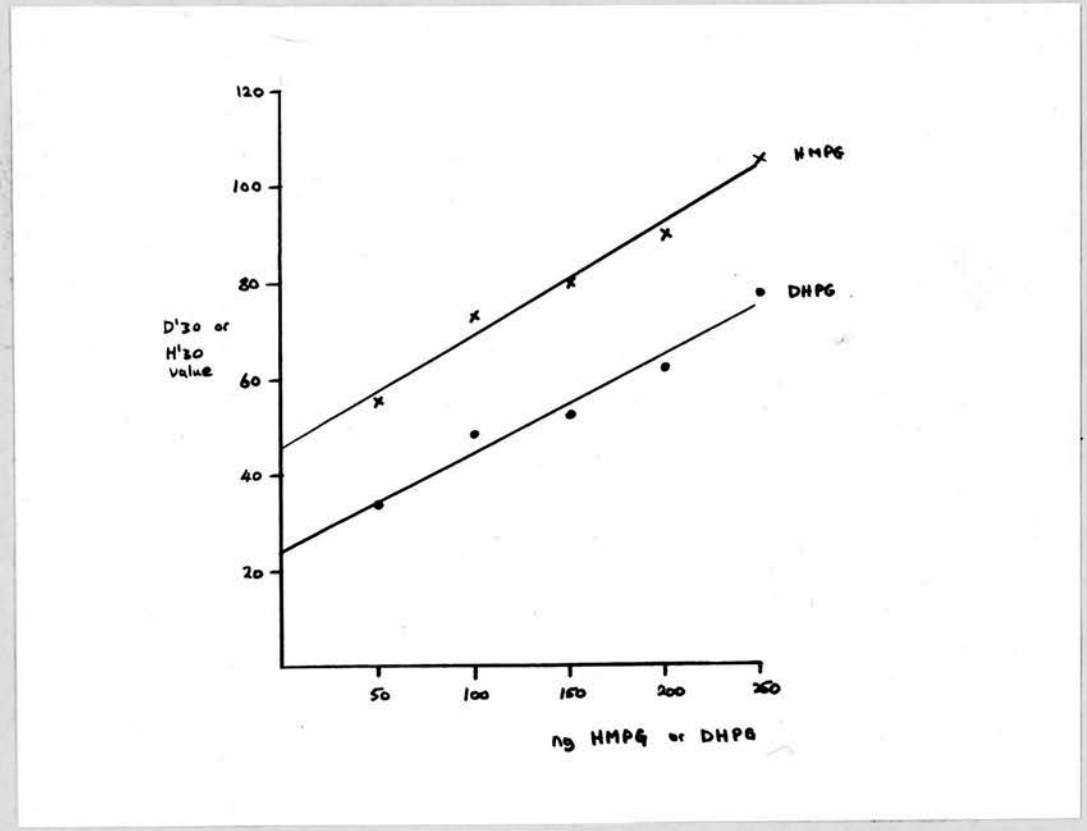

FIG. 20 The recovery of HMPG and DHPG added to the same aliquot of pooled rat brain tissue and taken through the method, modified for the simultaneous estimation of HMPG and DHPG, showing the linear relation between starting concentration of glycols and the H'30 or D'30 value. Each point is the mean of two determinations. The best straight lines were drawn from the original data by the method of least squares.

HMPG slope = 0.241

DHPG slope = 0.212

A comparison of the slopes by an analysis of covariance indicated that the difference between the slopes was not significant

(F ratio = 1.7419 $\gamma^1 = 1$ $\gamma^2 = 6$)

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TABLE 5 Mean ± SD concentration of total HMPG (free + conjugated) in whole rat brain, cerebral cortex, and cerebellum.

Area	Concentration of total HMPG ng/g mean ± SD	n
Whole brain	95 ± 41	21
Cerebral cortex	102 ± 26	65
Cerebellum	89 ± 30	10

measured using the modified method for the simultaneous extraction and derivative formation, are summarised in table 6. Both the original method for HMPG and the modified method gave similar values for the endogenous concentration of HMPG measured in whole rat brain and cerebral cortex.

The ratio of total HMPG to total DHPG in whole brain and cerebral cortex was 1:1.3 and 1:1.5 respectively, or expressed as a mean percentage difference of the metabolites, there was 38% more total HMPG than total DHPG in whole brain and 47% more total HMPG than total DHPG in cerebral cortex (Figs.21 and 22). This difference in the total (free and conjugated) concentration of total glycols was attributable to the fact that only trace amounts of free DHPG were detectable in both whole brain and cerebral cortex, whereas free HMPG was present in both, at concentrations between 20 ng/g and 26 ng/g, representing about one quarter of the total HMPG concentration. Figs.21 and 22 also show that in both whole brain and cerebral cortex, the ratio of the glycol sulphate concentrations was unity, a finding which agrees with a similar observation obtained from the intraventricular injection of (^{14}C)-DL-noradrenaline, followed by the isolation of the radioactively labelled conjugate fraction, where a ratio of unity for (^{14}C) HMPG-SO_4 and (^{14}C)-DHPG-SO_4 was also found (Fig.8).

TABLE 6 Mean ± SD concentrations of free and conjugated HMPG and DHPG in whole rat brain and cerebral cortex. *Free DHPG was found in only 3 samples from each group at the concentrations indicated.

Area	concentration ng/g mean ± SD n = 8					
	HMPG	HMPG-SO_4	Total HMPG	DHPG*	DHPG-SO_4	Total DHPG
Whole brain	23±5	58±10	84±14	5,3,3	56±8	57±8
Cerebral cortex	26±7	68±14	95±18	10,7,7	65±25	69±25

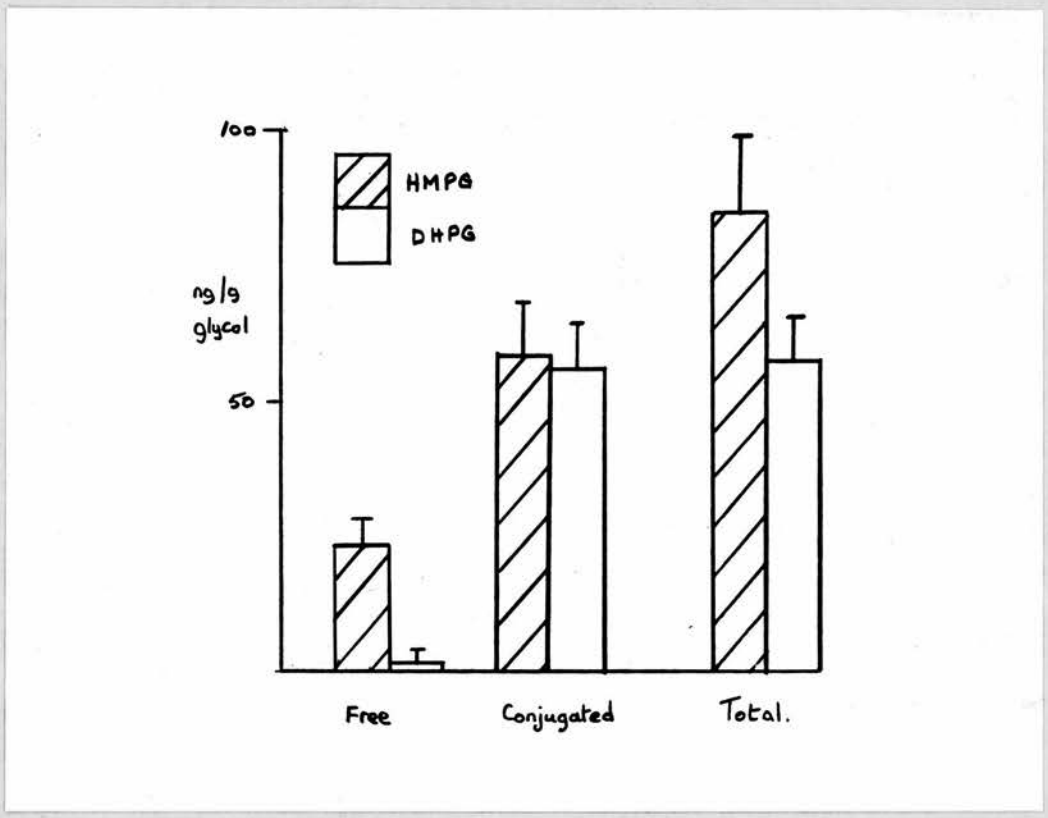

FIG.21 Histogram showing the relative proportions of HMPG and DHPG, free and conjugated found in whole rat brain. (Mean $\pm$ SD for 8 rats). No significant difference was found between the concentrations of HMPG-SO_4 and DHPG-SO_4 ($t = 0.41$) but there was 38% more HMPG than DHPG when expressed as free + conjugated.

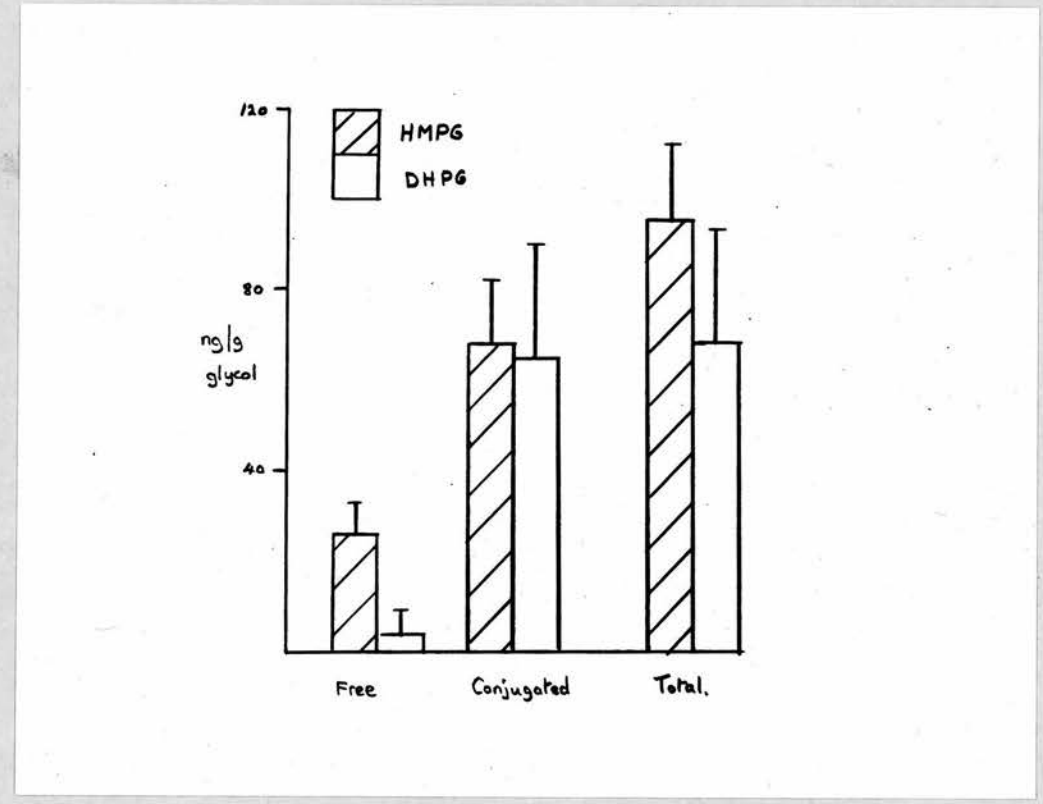

FIG.22 Histogram showing the relative proportions of HMPG and DHPG, free and conjugated found in rat cerebral cortex (mean ± SD for 8 rats). No significant difference was found between the concentrations of HMPG-SO_4 and DHPG-SO_4 ($t = 0.51$) but there was 47% more HMPG than DHPG when expressed as free + conjugated.

DISCUSSION

One part of this section describes the development of methods to allow the extraction and purification of HMPG and HMPG-SO_4 from rat brain, for quantitative analysis using the fluorimetric method of Antun et al (1971). There were two main problems with the fluorimetric method.

a) at the excitation and emission wavelengths used, there was a large fluorescence contribution to the blank value which originated from solutions and materials used in the purification procedure such as perchloric acid and potassium hydroxide, and more particularly 'Helicase' enzyme preparation. To overcome the blank contribution from perchloric acid and potassium hydroxide, a boiling water method was used to precipitate brain protein followed by a petroleum ether extraction, when blank values were obtained comparable to those obtained with distilled water alone. Some reduction of the impurity introduced with the 'Helicase' preparation was made using chromatography with 'Sephadex' LH20 gel or 'Bio-Rad' AG1x4 resin, but insufficient to give a reasonable blank value. Attempts were made to purify the enzyme preparation using dialysis or chromatography on 'Sephadex' G-200 gel, but both these methods were of no use since the preparation also contained an enzyme capable of digesting both the dialysis tubing and the 'Sephadex' gel. One procedure giving some purification was by making an acetone precipitate of the preparation, but this technique was not used because of the possible inconsistency in the enzymic activity after such treatment.

By investigating the hydrolysis rate characteristics of the 'Helicase' preparation, when applied to HMPG-SO_4 in rat brain, it was noticed that a reduction could be made to one fifth of the amount of enzyme normally used for the hydrolysis of HMPG-SO_4 in urine. Although this reduced the level of contamination considerably, the blank value through the method was still too great (Fig.11(c)) to allow the accurate estimation of small quantities ($<$ 200 ng) of HMPG.

b) The second problem with the fluorimetric method was that the sensitivity was too low. In pure solution, the lowest concentration of HMPG giving twice the blank value was 30 ng. Following the hydrolysis, extraction and purification steps, the sensitivity decreased to about 200 ng. Antun et al (1971) described a similar loss of sensitivity from 50 ng to 100 ng when the method was applied to the estimation of HMPG in urine. This was less important in their case, since there is a much greater concentration of HMPG in urine than in rat brain.

Various methods have been used for the quantitative estimation of HMPG by gas liquid chromatography. Wilk et al (1967, 1971 and 1972) and Dekirmenjian and Maas (1970) form a derivative of HMPG which has trifluoracetyl groups attached to the phenol group in addition to the two hydroxyl groups forming the glycol moiety, whereas Sharman (1969) Caesar and Sharman (1972) and Bond (1972) acetylate the phenol group prior to fluoracylation of the glycol. Dekirmenjian and Maas (1970) have criticised the method of

Wilk (1967) on the grounds that 'uncontrolled hydrolysis of conjugated HMPG and the use of an AG1x4 resin resulted in a 50% loss of HMPG which limits the reproducibility of the method' and describe simplifications in the technique, and data on the hydrolysis of HMPG which enhances the reproducibility and increases the recovery of the method. They also point out, however, that trifluoracetylation of the phenol group does not take place in totally anhydrous conditions, such as when the ethyl acetate is dried with phosphorous pentoxide. The relative hydration of the ethyl acetate may then be the critical factor in the reproducibility of the method. Acetylation of phenol groups, however, has been shown to proceed easily and quantitatively in aqueous solution (Welsh 1955, Crawford and Yates 1970). Sharman (1969) and Bond (1972) make use of this fact to overcome the problem of the fluoracylation of the phenol group, and also as an aid in the further purification of the glycol, acetyl-HMPG being readily extracted with the hydrophobic solvent 1:2 dichlormethane, thus bringing the metabolite into a phase suitable for the fluoracylation of the glycol-hydroxyl groups. Sharman (1969) used the anhydride of heptafluorobutyric acid to further acylate the glycol group. This acetyl-HFB derivative has the advantage of producing a much greater ECD response than the comparable acetyl TFA derivative used by Bond (1972), by virtue of the greater number of attached fluorine atoms. Certain problems, however, were experienced with the method of Sharman. Firstly, although the derivative could be formed

at all concentrations of acetyl-HMPG used, attempts to show a linear relation between the amounts of starting material and the electron capture detector response failed, and secondly, the fact that the anhydride had a high boiling point (108°C) made total removal of the reagent more difficult than with the lower boiling (40°C) trifluoracetic anhydride. Bond (1972) also expressed a preference for the formation of the acetyl trifluoracetyl derivative on the grounds that 'this appeared easier to form and once formed was very stable'. No difficulty was experienced in obtaining a linear relation between the amount of starting material and the ECD response of the acetyl-TFA derivative of HMPG formed as described earlier(Fig.16).

The gas chromatography method described here is at least fifty times more sensitive than the fluorimetric method of Antun et al (1971) permitting the accurate estimation of both free and conjugated HMPG in parts of brain weighing as little as 250 µg. Recently, however, a fluorimetric method for the estimation of HMPG-SO_4 as the conjugate, has been described (Meek and Neff 1972)which the authors claim is 'about as sensitive as gas chromatography methods but that it is more rapid and convenient'. This involves the isolation of the conjugate fraction on a column of 'DEAE Sephadex' after which it is eluted in a pure form and condensed with ethylene diamine in the presence of cysteine and perchloric acid at 100°C for 12 min, to form a fluorphore. The sensitivity of the method is about 12 ng, which amount of HMPG-SO_4 in pure solution gives a

fluorescence intensity twice the blank. This method has clear advantages since it eliminates the use of 'Helicase' enzyme preparation for the hydrolysis of the conjugate, so avoiding high method blank values, a factor which could account for its high sensitivity. Although the authors report that the fluorphore formation is very specific, the only other compound forming a fluorphore, amongst a whole list of catecholamine metabolites being free HMPG, they did not exclude the possibility that DHPG-SO_4 also forms a fluorphore. This is important since a preliminary evaluation of the use of the 'DEAE-Sephadex' column has shown that (^{14}C)-HMPG-SO_4 and (^{14}C)-DHPG-SO_4, prepared from (^{14}C)-DL-noradrenaline as described earlier, are eluted from the column in the same sample, if the procedure of Meek and Neff (1972) is followed. Similar values for the concentration of HMPG-SO_4 in rat brain to those reported here, however, have been obtained by the authors, and a later publication (Korf et al 1973) showing the effects of electrical stimulation and electrolytic ablation of the locus coeruleus on the concentration of HMPG-SO_4 measured by the fluorimetric method are consistent with results reported in Section II of this thesis and reported in the literature (Walter and Eccleston 1973, Arbuthnott et al 1973). This shows that their method is comparable to the method described here, but perhaps less versatile in its present form, since the gas chromatographic method is capable of measuring the concentrations of both free and conjugated HMPG and DHPG in parts of brain without loss of sensitivity.

Perhaps one criticism of the gas chromatography method is the low recovery, 100 ng of HMPG added to pooled rat brain having a mean percentage recovery of only 35.1 ± 3.3 (10) [mean ± SD (number of observations)] , but it was found reasonable to sacrifice recovery for purity and although the recovery was low, it was consistent, as shown by the low standard deviation about the mean. This consistency is also reflected in figs.17 and 20 which show the linear relation between starting amounts of HMPG and DHPG added to pooled rat brain and taken through the method, and the ECD response of the formed derivatives when adjusted with respect to hexachlorocyclohexane. It should be noted however, that greater recoveries could possibly be obtained if a pure form of sulphatase enzyme was available commercially since much of the purification procedure described here was concerned with the removal of impurities introduced with the addition of the crude sulphatase preparation 'Helicase'.

Reported values for the endogenous concentration of HMPG in brain are few. Schanberg et al (1968a) measured the free and conjugated glycol in the rat, guinea pig, cat, rhesus monkey and green monkey brain, whereas Sharman (1969) measured the concentration of free HMPG and DHPG in the hypothalamus of the rabbit, mouse and cat. More recently, Meek and Neff (1972) measured the sulphate ester of HMPG in whole rat brain and parts of brain, and Korf et al (1973) showed alterations in the endogenous levels of HMPG-SO_4 after electrical stimulation and electrolytic

ablation of the locus coeruleus. The reported values for rat brain are summarised in table 7 along with values reported in the results section.

The mean concentration of HMPG in rat brain was not significantly different when measured by the original method (table 5) or the method modified to permit the simultaneous measurement of DHPG (table 6). There was a similar concentration of total HMPG (free and conjugated) in whole brain, cerebral cortex and cerebellum. In whole brain and cerebral cortex about a quarter of this was consistently found as the free glycol (table 6). This was not the case with 3:4 dihydroxyphenol glycol, DHPG. Only trace amounts of free DHPG (3 ng-10 ng) were detectable in one or two of the nine samples derived from both whole brain and cerebral cortex, whilst the sulphate ester of DHPG was present in both preparations in concentrations equal to the concentration of HMPG-SO_4. This would immediately refute the idea that HMPG-SO_4 is the major endogenous metabolite of NA in rat brain, but since only small amounts of free DHPG can be demonstrated, the concentration of total (free + conjugated) HMPG is greater than the concentration of total DHPG.

Various reasons may be made to explain why HMPG should be present in the free and conjugated form, whilst DHPG is found mostly as the conjugate.

a) One could consider that both glycols have equal access to the sulphating enzyme system, but because HMPG has a

TABLE 7 Mean concentration of HMPG and HMPG-SO_4 in rat brain found by various workers, compared to the results obtained here. Values, although in some cases are more than double, are of the same general order, falling within the range of 50-130 ng/g for HMPG-SO_4.

Area		Schanberg et al (1968a) ng/g	Meek & Neff (1972) ng/g	Korf et al (1973) ng/g	This Thesis ng/g)
Whole Rat Brain	Free	0	-	-	23
	Conjugated	50	130	-	58
Cerebral Cortex + Hippocampus	Free	-	-	-	26
	Conjugated	-	76	114	68

3-methoxy group in place of a hydroxyl group, this results in steric hindrance, causing only the partial sulphation of HMPG.

b) Alternatively one could consider that the two glycols have unequal access to the sulphating enzymes. One reason for this could be that if COMT had an extraneuronal location, the 3-methoxy derivatives might then be less actively taken up into the (nerve) cells than their 3:4 dihydroxyphenyl counterparts (Iversen 1966). If this reasoning was correct one might expect that electrical stimulation of NA nerves would produce a greater increase in free HMPG than in conjugated HMPG. This, however, does not appear to be the case for stimulation of the locus coeruleus. Fig.31 in Section II shows that electrical stimulation of this nucleus produces an increase in HMPG-SO_4 with no significant change in free HMPG, when whole brain is examined.

c) One explanation for the very low level of free DHPG compared to free HMPG could possibly be made considering the results of Eccleston and Ritchie (1973). They showed that when DHPG was injected into the lateral ventricle of the rat along with labelled sulphate, 37% of the radioactivity appeared as HMPG-SO_4 on subsequent analysis, indicating a rapid methylation of DHPG prior to sulphation.

More experimentation will be necessary to fully explain these phenomena, as well as the observations by other workers that in some species such as the rabbit and the cat, sulphation of HMPG would not appear to be a normal metabolic route (Mannarino et al 1963).

In summary, this section describes the development of a method for the quantitative estimation of HMPG and HMPG-SO_4 in brain tissue, and the later modification of the method to permit the simultaneous estimation of DHPG and DHPG-SO_4. The method is very sensitive, allowing parts of rat brain to be analysed accurately without loss of sensitivity, and giving results for the concentration of HMPG and HMPG-SO_4 not at variance with previously reported values. The techniques are applied in the next section to a study of the effect of the physiological manipulation of a NA-containing neuronal system in the brain, that from the locus coeruleus to the cerebral cortex.

SECTION 2

THE EFFECT ON THE METABOLISM OF NORADRENALINE IN RAT BRAIN OF THE ELECTROPHYSIOLOGICAL MANIPULATION OF THE NUCLEUS LOCUS COERULEUS

INTRODUCTION

Following the discovery that the catecholamines noradrenaline and dopamine, and the indolamine 5-hydroxytryptamine could be demonstrated biochemically in brain tissue (Vogt 1954, Bertler and Rosengren 1959, Amin et al 1954, Carlsson et al 1958, Montagu 1957), where they occurred with unequal distributions, methods were sought to reveal their cellular location.

The observation that tissue containing biogenic amines became highly fluorescent when fixed in formalin led Eränko in 1955 to attempt to use this fluorescence as a qualitative measure of noradrenaline and adrenaline in situ in the adrenal medulla of the cat, from which he concluded that 'noradrenaline is responsible for the fluorescence of the medullary islet ..... which became visible only if the sections are treated with formalin'. The use of formalin, however, had great disadvantages, since NA was readily soluble in the fixative making the technique no use for demonstrating NA in sympathetic nerves, where the concentration of the amine was relatively small and the possibility of diffusion, great. By freeze-drying the tissue and treating with formaldehyde vapour from paraformaldehyde at a specific humidity, greater control and sensitivity was achieved for the production of the fluorescent derivatives of the amines (Falck et al 1962, Falck 1962). Simultaneously, the Swedish workers applied their techniques to the study of the brain, looking first at the hypothalamus. Here they found much evidence of fine structure exhibiting

the green to green-yellow fluorescence which is characteristic of material containing catecholamines (Carlsson et al 1962). Along with the histochemistry they used amine depleting agents such as reserpine, and synthesis inhibitors such as meta-tyrosine and α-methyl meta tyrosine to confirm the identity of the particular amine made visible in this area. They concluded that the abundant fluorescent varicose fibres present were terminal structures from adrenergic fibres in the brain. This prompted a great deal more research on the amine containing systems present in brain and led to the discovery of discrete groups of nerve cell bodies in the brain stem which became fluorescent after treatment with formaldehyde (Dahlstrom and Fuxe 1964). These were of two distinct types, those showing a weak to strong green fluorescence in normal animals and animals treated with monoamine oxidase inhibitors, and those showing a very weak yellow fluorescence in untreated animals, which had a medium to strong yellow fluorescence after the administration of monoamine oxidase inhibitors to the animal. The authors attributed these cell types to catecholamine and 5-HT containing cells respectively. They went on to describe the occurrence of four groups of each cell type in the medulla oblongata, three groups of each type in the pons, three groups of each type in the mesencephalon and two groups of catecholamine containing cell bodies in the diencephalon. As well as noradrenaline, dopamine also occurs in brain in concentrations large enough to be responsible for some of the green fluorescence noted

by Dahlstrom and Fuxe (1964) (Carlsson 1959, Bertler and Rosengren 1959). Also, the distribution of DA in brain does not parallel the distribution of NA, indicating that dopamine is not just a precursor of noradrenaline, but has some separate function. The main areas of high DA content are the caudate nucleus and putamen, and the mesencepalan (Carlsson 1959). It was shown later that in the human brain one area with a fairly high dopamine content was the zona compacta of the substantia nigra (Hornykiewicz 1963) and that the dopamine in the neostriatum occurs in very high concentrations in nerve terminals which arise from the cells of the substantia nigra region (Anden et al 1964). The lower brain stem, that is the pons and the medulla oblongata has a very low DA content compared to the concentration of noradrenaline, indicating that the green fluorescent cells of the catecholamine type found in this area are more likely noradrenaline containing. The most conspicuous fluorescent cell body group in this area is the group A6 (in the nomenclature of Dahlstrom and Fuxe 1964) which occurs in the pons and which the authors describe as 'quite unique as regards its cell composition, practically all of its closely packed nerve cells belong to the catecholamine type. This group seems to be identical with the locus coeruleus'.

Using the same fluorescence histochemical technique used for the demonstration of nerve cell bodies in brain, Fuxe (1965) described the distribution of catecholamine-containing and 5HT-containing nerve terminals in rat brain.

The problem of distinguishing between DA-containing and NA-containing terminals was again approached with the use of the catecholamine synthesis blocker α-methyl meta tyrosine (αMMT). This drug blocks the resynthesis of catecholamines by competitive inhibition of the enzyme tyrosine hydroxylase, causing a reduction in tissue fluorescence. Carlsson 1964 and Fuxe 1965b have reported that the recovery of fluorescence following a dose of αMMT is faster with dopamine containing terminals than with noradrenaline terminals and this can therefore be used as a diagnostic tool. Using this sort of classification, Fuxe found, in the lower brain stem, high densities of NA-nerve terminals in many of the visceral afferent and efferent nuclei such as the nuclei of the solitary tract, the dorsal motor nucleus of the vagus nerve, the motor nucleus of the vagus nerve and the motor nucleus of the trigeminal nerve, in the pallidus and obscura nuclei of the raphe system, in parts of the reticular system and in the ventrolateral grey matter of the mesencephalon, but the majority of lower brain stem regions showed low density of catecholamine-terminals, and in some regions such as the nucleus ruber and large parts of the substantia nigra, no catecholamine nerve terminals were apparent using this technique.

The hypothalamus had the highest density of NA-terminals in the diencephalon, whilst in the remainder of the thalamus only a scattered low density of terminals, or none at all, were seen, with the exception of two nuclei, the anterior ventral thalamic nucleus and the nucleus paraventricularis

rotondocellularis, both of which had a very high density of terminals. NA-terminals were present in most parts of the telencephalon with considerable numbers being found in the hippocampus, the amygdaloid cortex and the gyrus cinguli and in the ventral part of the interstitial nucleus. The neocortex and olfactory cortex contained a uniform distribution of NA-terminals of medium to low density.

Dopamine nerve terminals were present in defined areas such as the neostriatum, the olfactory tubercle and the nucleus accumbens, with a high density in the median eminence.

The origin of catecholamine nerve terminals has been shown to reside in the catecholamine containing nerve cell bodies previously described (Dahlstrom and Fuxe 1964) which occur in the mesencephalon and the lower brain stem. Most of the work concerned with mapping out these neuronal systems has been by making lesions at definite levels of brain followed by examination of histological sections rostral and caudal to the lesion for the accumulation of fluorescent material, and also by examination of areas rich in catecholamine terminals for changes in fluorescence. Using these techniques, Ungerstedt (1971) was able to confirm earlier work which showed that ascending catecholamine and 5HT pathways entered the medial forebrain bundle (Anden et al, 1966; 1967, Dahlstrom et al 1964, Loizou 1969), that there was a DA-containing neuronal pathway from the substantia nigra to the corpus striatum (Anden et al 1964, Hökfelt and Ungerstedt 1969) and that neuronal pathways

from the locus coeruleus gave rise to terminals having a widespread distribution in the brain (Loizou 1969) and more specifically in the cerebellum (Olson and Fuxe 1971). Ungerstedt further showed that the locus coeruleus gave rise to three catecholamine-containing pathways, a lateral pathway to the cerebellum, which enters via the medial cerebellar peduncle, a descending pathway which innervates the lower brain stem and probably makes synaptic contact with the lower brain stem NA-containing nuclei, and a major ascending pathway described by Ungerstedt as the dorsal NA pathway. This pathway initially forms the dorsal part of the large bundle of NA-containing axons in this region but 'at the caudal level of the nucleus pontis, the axons turn dorso-medially to form a completely separated dorsal bundle of axons' (Ungerstedt 1971) (Fig.23). At the rostral level of the nucleus mamillaris, the axons of the dorsal bundle turn ventrolaterally to rejoin the other ascending NA and DA axons (Fig.24). The axons then ascend in the medial forebrain bundle and in the septum and turn caudally in the cingulum.

Lesions of the locus coeruleus and the dorsal bundle showed that this pathway innervates the cortex and the hippocampus although the way of entry of the bundle into these areas is obscure since lesions of the rostral hypothalamus were not as effective in denervating the cortex as lesions of the dorsal bundle at the level of the mesencephalon.

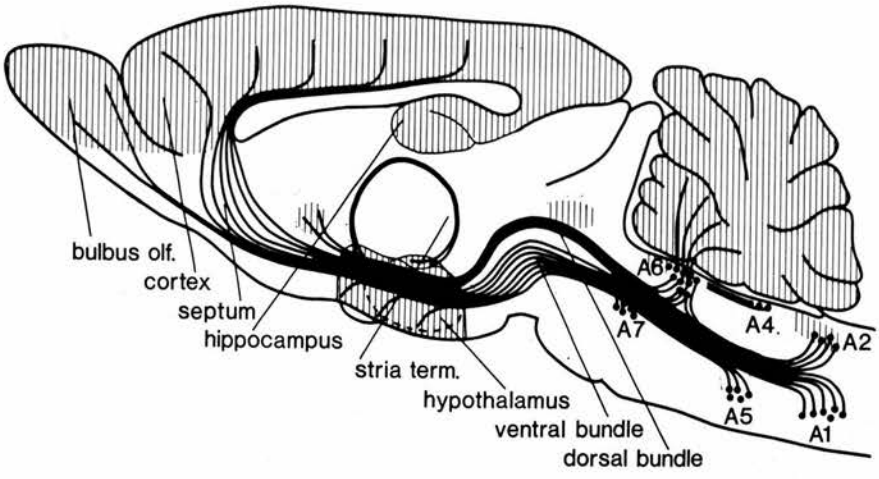

FIG.23 Sagittal projection of the ascending NA pathways. The stripes indicate the major nerve terminal areas. A6 is the locus coeruleus using the nomenclature of Dahlstrom and Fuxe (1964). Figure taken from Ungerstedt (1972).

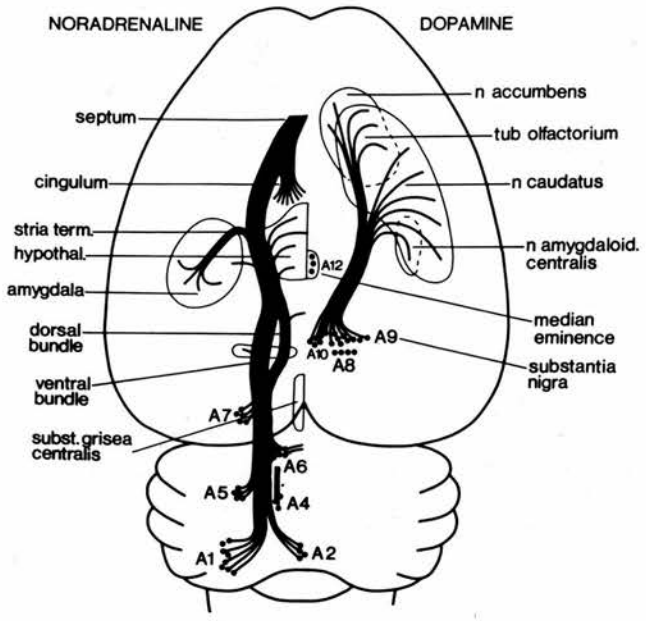

FIG.24 Horizontal projection of the ascending NA and DA pathways. A6 is the locus coeruleus, using the nomenclature of Dahlstrom and Fuxe (1964). Figure taken from Ungerstedt (1972).

Nerve terminals arising from the locus coeruleus were found in practically all areas of the brain but especially in the neocortex the hippocampus and the cerebellar cortex.

The existence of catecholamine and 5HT-containing pathways in the brain has been confirmed by studying the biochemical effects of making specific lesions, or by electrical stimulation of the cell bodies and axonal pathways of these systems. Lesions made in the ventromedial tegmental area have been shown to cause a reduction in the dopamine content of the basal ganglia and an alteration in the activity of some catecholamine synthesising enzymes. (Poirier et al 1965, Sourkes 1966, Goldstein et al 1969) and electrical stimulation in the region of the substantia nigra caused the release of dopamine from the putamen (McLennan 1965, Portig and Vogt 1969).

Electrical stimulation of the midbrain raphe system has been shown to give rise to an increase in the turnover of brain 5HT (Aghajanian et al 1967, Sheard and Aghajanian 1968, Kostowski et al 1969, Shields and Eccleston 1972) and lesions of the medial forebrain bundle, the raphe area, the hypothalamus and the septum caused reductionsin the level of 5HT in the brain (Harvey et al 1963, Jouvet et al 1966, Kostowski et al 1968, Parent and Poirier 1969).

The effect of lesions on brain noradrenaline concentrations is not so well defined. Heller and Moore (1965) and Moore et al 1966 showed that lesions in the region of the hypothalamus caused a reduction in brain NA and dopa decarboxylase activity, and Sourkes (1966) found an

ipsilateral decrease in DA and NA in the caudate and lenticular nuclei after a unilateral lesion of the ventromedial tegmental area of the upper pons and midbrain, whilst Benetato et al (1967) showed that lesions of the medial forebrain bundle caused no reduction of noradrenaline and adrenaline in the rat hypothalamus, amydala and hippocampus. Also, Parent et al (1969) could show no difference in the hypothalamic NA concentration using a series of lesions in the region of the medial forebrain bundle although the 5HT concentration could be ipsilaterally reduced when the lesion was of the medial forebrain bundle.

Arbuthnott et al (1970) showed that by stimulating the noradrenaline containing pathways in the brains of rats pretreated with NA synthesis inhibitors, a depletion of catecholamine fluorescence could be obtained in areas of brain rich in NA terminals, and that the distribution of this depletion of fluorescence was different after stimulating the 'dorsal pathway' from the locus coeruleus, from that obtained after stimulating the ventral NA-pathway arising from the other NA brain stem nuclei. They showed that 'dorsal pathway' stimulation caused a more rapid depletion of catecholamine fluorescence in the hippocampal formation, the neocortex and the gyrus cingulum, without any increased rate of depletion in the hypothalamus, the preoptic area, the nucleus interstialis striae terminalis and the ventral part of the limbic system. Stimulation of the 'ventral pathway', however, caused a more rapid depletion of fluorescence in the hypothalamus, the preoptic

area, the amygdaloid cortex and the septal area on the stimulated side with no observable effects in the neocortex the cingulum and the hippocampus.

Two groups have put forward possible explanations of the function of the very diffuse neuronal system which has its cell bodies in the locus coeruleus and axon projections which terminate in all cortical areas of brain (and possibly also hypothalamus), these explanations based on observations they have made of alterations in the behaviour of animals which had had either lesions of the system, or had chronically implanted electrodes and had been trained to stimulate themselves.

Jouvet and his group made bilateral lesions of the locus coeruleus and the raphe nuclei in cats to discover the importance of these nuclei in sleep mechanisms. From their results they postulated that the control of total atonia in paradoxical sleep is performed through the locus coeruleus since cats with bilateral locus coeruleus lesions could not achieve atonia although they showed the normal electrical effects of paradoxical sleep.

On the other hand, Crow et al 1972 have shown that rats with chronically implanted electrodes with tips at the locus coeruleus can be trained to repeatedly press a lever which delivers to them a train of stimuli, a type of positive reinforcement behaviour described earlier by various workers who made more rostral placements of electrodes. (Olds and Milner 1954, Olds et al 1960, Wilkinson and Peele 1963, Valenstein 1966). Self stimulating behaviour was also

shown to be supported in the region of the superior cerebellar peduncle (O'Donahue and Hagamen 1967, Routtenberg and Malsbury 1969) and in some parts of the floor of the fourth ventricle (Routtenberg and Malsbury 1969). Crow (1972) also demonstrated that electrical self stimulation in the ventromedial tegmental area of the mesencephalon was associated with electrode tips at dopamine containing cell bodies (groups A8, A9 and A10 of Dahlstrom and Fuxe 1964). When previous results describing areas of self stimulating behaviour are compared with the more recent detailed maps of catecholamine-containing neuronal systems (Ungerstedt 1971), there is a good correlation of sites of self stimulation with the catecholamine containing systems. Crow (1973) has therefore suggested that electrical self stimulation in brain is associated only with these systems, and on the basis of the anatomical nearness of central olfactory connections and central gustatory connections with the DA-containing and NA-containing systems respectively, has suggested that the latter systems are involved in 'incentive motivational' and 'reinforcement' components of the animals exploratory drive. These behavioural aspects are discussed more fully later in the light of results obtained in this section.

The work in this section describes the effect on the total (free + conjugated) HMPG concentration in rat brain of

a) acute electrical stimulation of the locus coeruleus under halothane anaesthesia;

b) after electrolytic ablation of the locus coeruleus and c) after conscious electrical self stimulation in animals having chronically implanted electrodes with the electrode tip at the locus coeruleus.

A preliminary report on the effect of electrical stimulation on the relative concentration of HMPG and DHPG in rat cerebral cortex is also included.

METHODS

Stereotaxic location of the locus coeruleus

a) Surgical techniques

Crow et al (1972) have used the following stereotaxic co-ordinates for the location of the locus coeruleus, using animals 180-200 g in weight:-

anterior posterior co-ordinate (AP) 1.8 mm posterior to the apex of the lambda suture in the mid line,

lateral co-ordinate (L) 1.0 mm lateral to the mid line,

vertical co-ordinate (V) 6.5 mm from the surface of the skull, when the skull surface between the lambda and bregma sutures was in a horizontal plane.

To facilitate reproducibility, it was decided to define the (AP) co-ordinate in terms of the ear bar zero and the (V) co-ordinate with respect to the position of the upper incisor bar set at 2.4 mm below the interaural line (as in König and Klippel 1963). Histological analysis of the electrode tip position showed that with rats 180-200 g, there was a greater than 60% chance of the electrode tip being at the locus coeruleus when the following stereotaxic co-ordinates were used:-

(AP) 1.7 mm posterior to the ear bar zero

(L) $\pm$ 1.0 mm to the mid line suture

(V) 6.5 mm below the skull surface.

Much less success was obtained with the co-ordinates of Crow et al (1972), probably because of slight variation in the position of the lambda apex.

The ear bar zero position for the (AP) co-ordinate was obtained as follows:- the electrode was clamped in the electrode holder, and after checking that it was vertical, the tip of the electrode was located at the point of a sharp ear bar clamped in position in the frame. This vernier reading was the (AP) zero position from which the required (AP) co-ordinate vernier reading was calculated. The electrode holder and ear bar were then removed from the frame.

Rats weighing between 180 and 200 g were placed in a plastic box, through which was passed 1.6% halothane in oxygen to render the animals unconscious, after which they were transferred to a 'David Kopf' stereotaxic frame (No. 1530) fitted with a rat incisor bar adapter, set at 2.4 mm below the interaural line. The head of the rat was fixed in position by inserting ear bars into both external auditory meata (sharp ear bars for acute experiments and 45° tip bars for recovery experiments, the latter used to prevent the puncture of the ear drum). The upper incisors were then hooked over the incisor bar, and the nose held in position with the screw clamp. The animal was maintained under anaesthesia by a supply of 0.8% halothane in oxygen delivered through a plastic bag fitted over its nose (Fig.25). A mid-line incision was made to reveal the skull surface about 0.8 mm anterior and posterior to the lambda suture, clearing the overlying connective tissue. The electrode holder was placed in the frame and the tip of the electrode located just above the mid-line suture of the skull, to

FIG.25 Plate showing the experimental procedure for stereotaxic surgery with the rat in the frame and the electrode in position. Anaesthetic was delivered to the rat through the plastic bag over the rat's nose.

give the (L) co-ordinate zero position, from which the (L) co-ordinate was calculated. This method has been shown (Shields 1972) to locate the mid line more accurately than the alternative method of centering the rat in the stereotaxic frame. Using the (AP) and (L) co-ordinates, the electrode tip was located at the position for entry and lowered to just touch the skull surface. This gave the (V) co-ordinate zero reading from which the (V) co-ordinate was calculated. The position for entry was marked on the skull and a 2 mm diameter hole drilled in the skull using a dental drill fitted with a BS 6 burr, the hole being sealed with sterile bone wax. The electrode tip was once more located at the point of entry using the (AP) and (L) co-ordinates and then lowered through the bone wax into the brain to the calculated depth.

The stimulating or coagulating current was then passed as described later, after which the electrode was withdrawn from the brain and the electrode holder removed. During this procedure the temperature of the anaesthetised rat was maintained at 37°C using a thermal blanket connected to a probe inserted in the rectum of the rat.

Stimulated animals were removed from the frame, decapitated and the brains carefully removed for biochemical and histological analysis.

The scalp wound of recovery animals was dusted with antibiotic powder (Polybactrin, Calmic Ltd.), the wound closed with Michel clips (12 mm, Martin) and the animals placed in a clean cage under infra-red illumination to

maintain body temperature between 37°C and 38°C for 1 hour postoperative. Clips were removed 10-14 days post-operatively under light halothane anaesthesia. In uni-laterally ablated rats, no abnormal behaviour was noticed, and animals after a brief pause (2 days) continued to gain weight at the normal rate. Two rats in the group with bilateral lesions, however, died shortly after recovery from anaesthesia, and it is also of interest to note that the remaining four rats showed a striking behavioural syndrome of marked hyperkinesia and repeated jumping which appeared within two hours after recovery from anaesthesia and lasted for four hours. (This behaviour with bilater-ally ablated rats has, however, not been repeated with further groups of rats used for other purposes. There has also been no further postoperative mortality.) Three weeks postoperative the rats were killed by cervical fracture and the brains carefully removed for histochemical and biochemical analysis.

For recovery experiments in which the electrode was permanently implanted in the brain ('self-stimulating rats'), male Hooded Lister rats of the same weight range were used, and the surgical procedure modified in the following way:- In addition to the burr hole through which the electrode would pass, five more holes were drilled and tapped, into which 6-BA nylon screws were fixed. These screws served as anchor points for the dental cement. The electrode, mounted in an 'ITT Cannon SA 2P' plug which allowed for ease of electrical connection post-operative, was inserted

in position in the brain so that the tip was at the locus coeruleus. With the temporalis muscle on each side of the skull retracted, dental acrylic cement was poured around the electrode assembly and nylon screws. When firmly set the retracted muscles were released, positioned, and after dusting the wound with antibiotic powder, the wound was sewn up. Following recovery, animals were placed in a standard operant conditioning apparatus designed to train the animals to stimulate themselves by pressing a lever, this described later.

After a period of self stimulating behaviour, rats were killed by cervical fracture, and the brain carefully removed for biochemical and histochemical analysis.

b) Histochemical methods

Histological analysis was made by using either a modification of the Klüver Barrera (1953) technique, or by making frozen serial sections and staining with toluidine blue.

The modified Klüver Barrera technique

With the dorsal surface of the brain uppermost, the brain stem and cerebellum was dissected from the brain by making a vertical cut in the frontal plane at the level of the posterior colliculus. The brain stem and cerebellum were fixed by placing in a 10% solution of Formal Saline for at least 2 days, after which the tissue was dehydrated by immersing for 12 hours each in 50%, 70%, 90% and absolute ethanol (2 times). The tissue was then cleared

in chloroform and vacuum embedded in paraffin wax at 54°C. Ribbons of sections were cut using a standard microtome and floated onto microscope slides precoated with a thin film of albumin in glycerol. Staining was with Luxol fast blue and Cresyl violet performed as follows:- sections were freed from paraffin wax with xylene for 10 min and partially rehydrated using absolute ethanol and 90% ethanol for 5 min each. The sections were stained with Luxol fast blue for 1 hour at 57°C (0.1% Luxol fast blue in absolute ethanol containing 5 ml of 10% acetic acid per litre). Sections were then further rehydrated by immersing in 70% ethanol and distilled water for 5 min each. Differentiation of the Luxol fast blue stain was made by alternate immersion in 0.05% lithium carbonate solution (5 min) and 70% ethanol (5 min) until satisfactory (usually twice).

Counterstaining was then made with 0.1% cresyl violet in 70% ethanol at 37°C for 10 min, and this further differentiated using absolute ethanol. The doubly stained sections were cleared in xylene and mounted with canada balsalm in xylene.

This method had the advantage that it revealed nerve cells and fibre tracts, and every section was available for examination, enabling the exact location of the electrode tip to be accurately determined. The method was, however, time consuming when compared to the cryostat sectioning technique coupled to toluidine blue staining.

Cryostat sectioning and toluidine blue staining

This method had the advantage that it was very quick and produced little distortion of structure, but had the disadvantage that prolonged storage of tissue prior to sectioning was not possible.

With the dorsal surface of the brain uppermost, a vertical cut in the frontal plane was made at the level of the posterior colliculus, and the brain stem with overlying cerebellum cooled at -20°C for 5-10 min until firm. A second vertical cut in the frontal plane was then made at a level just caudal to the paraflocculus. This gave a slice of tissue about 5 mm thick, which was placed with the posterior frontal plane flat against a cork disc, lubricated with a drop of water and the cork disc fixed onto a microtome chuck by rapid freezing using a carbon dioxide expansion device (South London Electrical Equipment Co.). The chuck was fixed onto the microtome (Pearse cold microtome type H) maintained at -20°C and serial frozen sections 15 μ thick taken for staining.

Automatic staining of the sections was made using toluidine blue as follows:- After fixing the sections by suspension over a 70% formaldehyde solution for 5 min the sections were successively immersed in;

(a) 100% ethanol for 1 min
(b) 50% ethanol for 2 min
(c) distilled water for 2 min
(d) 0.2% aqueous solution of toluidine blue for 5 min
(e) 50% ethanol for 2 min

(f) 100% ethanol for 2 min

(g) xylene for 2 min.

The sections were then mounted with canada balsalm in xylene. (Laszlo, unpublished method)

Electrical stimulation of the locus coeruleus

a) Choice of electrodes

Shields and Eccleston (1972) used concentric bipolar electrodesfor the electrical stimulation of the midbrain raphe nucleus. These were constructed by cementing a 0.12 mm varnished steel wire (Johnson Matthey Metals Ltd.) into stainless steel tubing 0.38 mm outside diameter using 'Araldite' varnish No. PZ 985. The outside of the electrode was insulated with 'Bakelite' lacquer No. L3128, and connecting wires soldered on with 'Arax' acid cored solder. The central wire was allowed to project 1 mm beyond the tube and 0.4 mm of this was scraped clean. 0.5 mm of the end of the outer tube was also scraped clean to allow current to flow between outer and inner portions. The electrodes generally had a resistance of between 30 and 50 K ohms in 1% saline.

Although these electrodes were effective in stimulating the locus coeruleus, causing an increase of HMPG (free + conjugated) in whole brain and in the cerebral cortex (see results, this section), the effect in the cerebral cortex was always bilateral, that is, both the cortex on the stimulated and opposite sides had similar raised levels of metabolite. Since Ungerstedt (1971) has shown that the

pathway from the locus coeruleus to the cerebral cortex is largely uncrossed, it was thought that the rise of metabolite level on the opposite side could be due to the simultaneous stimulation of the opposite nucleus locus coeruleus by spread of stimulating current. If the stimulating current was more localised, a unilateral rise of HMPG might occur. Unipolar electrodes, therefore, were used made from stainless steel entymological pins, 0.3 mm in diameter coated with 'Bakelite' lacquer No. L 3128 and the tip scraped clean. An indifferent electrode was clipped to the scalp wound.

b) Stimulating current

An identical circuit to the one used by Shields (1972) for stimulating the mid brain raphe system was used to electrically stimulate the locus coeruleus. Alternating pulses of constant current, were produced by passing through a high resistance circuit, 50-70 volt pulses from two 'Devices' Isolated Stimulators (Type 2533) connected in series, and triggered alternately by a 'Devices' Digitimer (Type 3290), current strength being monitored by displaying the voltage developed, across a resistor, on an oscilloscope (Tektronic 502A). A condensor (1 uF, 250 V) was also included in the circuit shown in Fig.20, to eliminate any net current due to slight imbalance between the stimulators. The square wave pulses were 2 msec duration and at a current strength of 0.2 mA and a frequency of 10 Hz except where stated otherwise.

The following optimum parameters for stimulation, with respect to the rise in the level of total HMPG (free +

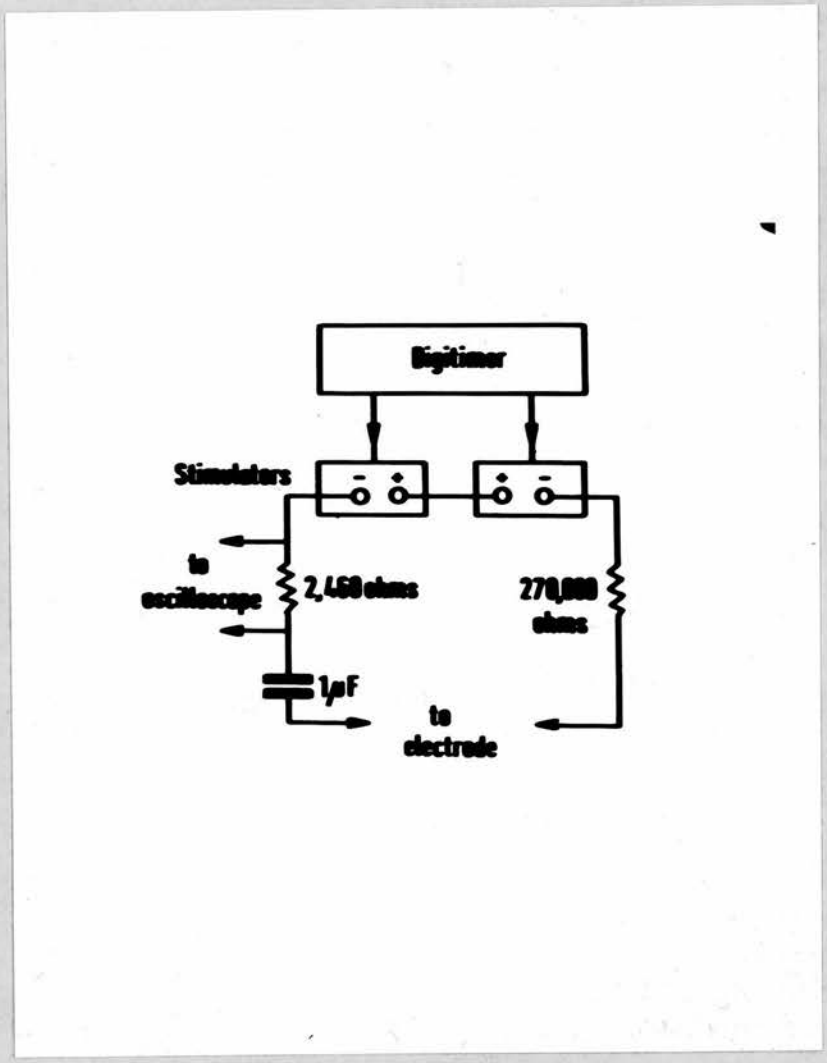

FIG.26 Electrical circuit used for stimulation experiments.

conjugated) were measured:-

(a) The effect of the frequency of stimulating pulses

(b) The effect of the current strength of stimulating pulses, using concentric bipolar electrodes and unipolar electrodes.

The time of stimulation in all experiments was 45 min.

Method for the production of self-stimulation with electrodes in the region of the locus coeruleus.

a) Electrodes

Two strands of 60 µm 'Teflon'-coated stainless steel wire were twisted together to form a bipolar electrode. The electrode was then mounted in a special plug assembly (ITT Cannon SA2P) which contained sockets for the supply leads.

b) Circuit (Fig.27)

The rat was placed in a standard operant conditioning apparatus (Foringer Apparatus Inc.) (Fig.28) at one side of which was a lever placed 8 cm from the floor of the cage. Depression of the lever triggered the release to the animal through the stimulating electrode, of a 200 msec train of 100 H_z unidirectional pulses of 0.5 msec duration from a 'Devices' isolated stimulation unit. A 200 msec gate was included in the circuit to ensure that a full 200 msec pulse was delivered before further triggering occurred. A voltage step on the isolation unit provided the means for increasing the current strength, the value

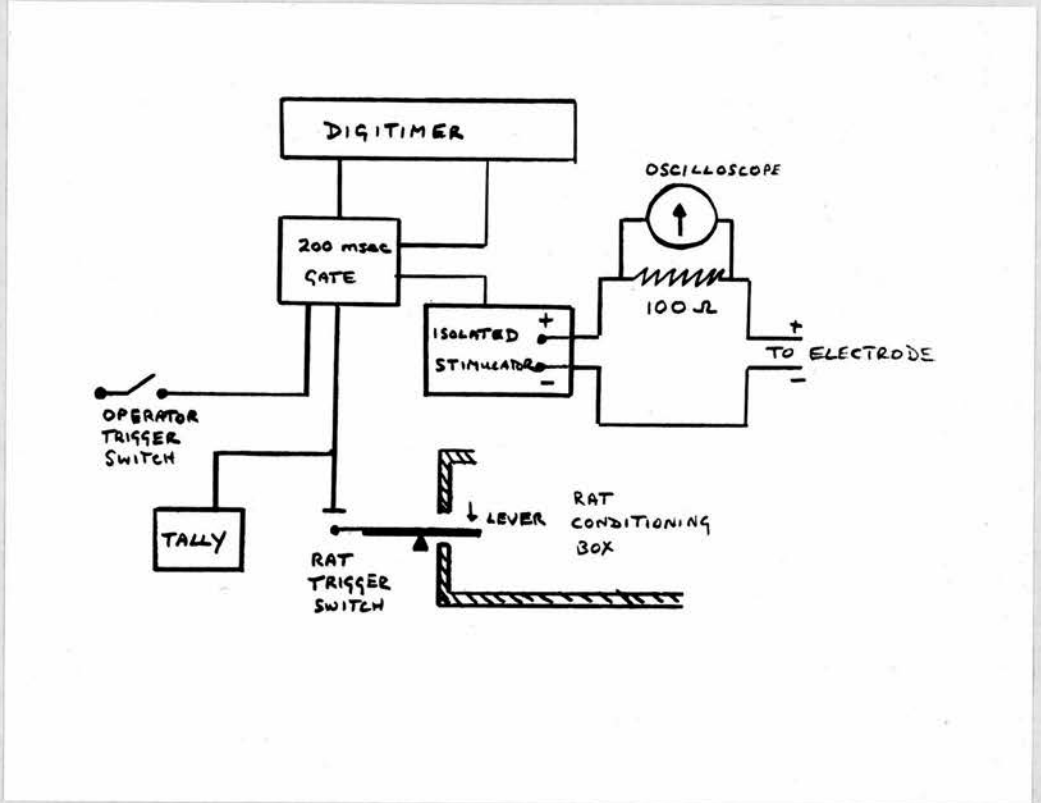

FIG.27 Electrical circuit used for self stimulation experiments showing the facilities for operant training.

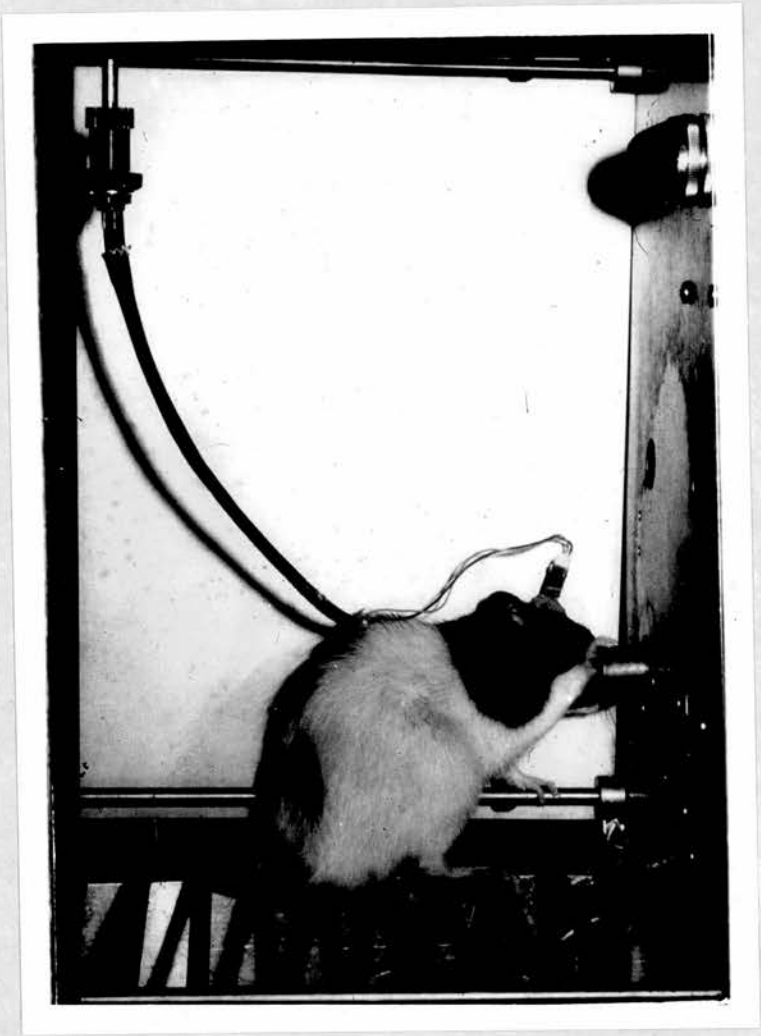

FIG.28 Plate showing a rat in the operant conditioning apparatus in the process of pressing the lever, to receive an electrical stimulus, via the leads, to the electrode assembly mounted on it's head.

of which was measured by a meter in circuit.

Rats were trained to respond during three test sessions of at least 30 min each, when the rat was introduced to the lever, and trained to press it, depression of which caused electrical stimulation of the area of brain surrounding the electrode tip. The current strength was increased in successive 25 μA steps until lever pressing was established. The response rate to classify the animals as positive for electrical self-stimulation, was at least 500 lever depressions per hour over two 15 min test periods.

Electrolytic ablation of the locus coeruleus

Unipolar electrodes of the type used for electrical stimulation, were used to cause an electrolytic lesion in the brain at the electrode tip. A simple circuit was needed for this procedure (Fig.29). A 12 volt battery eliminator was used to give a direct current supply, which passed from the positive pole, through a meter and a switch to the electrode, an indifferent electrode clipped to the scalp wound completing the circuit. As the current varied between 50 μA and 300 μA, depending on the state of the electrode, the pulse time was adjusted so that a total charge of 6 millicoulombs was passed.

Quantitative estimation of HMPG and DHPG

The concentrations of HMPG and in some experiments the ratio of HMPG to DHPG, were measured, after stimulation or ablation of the locus coeruleus, using the techniques described in Section I of this thesis.

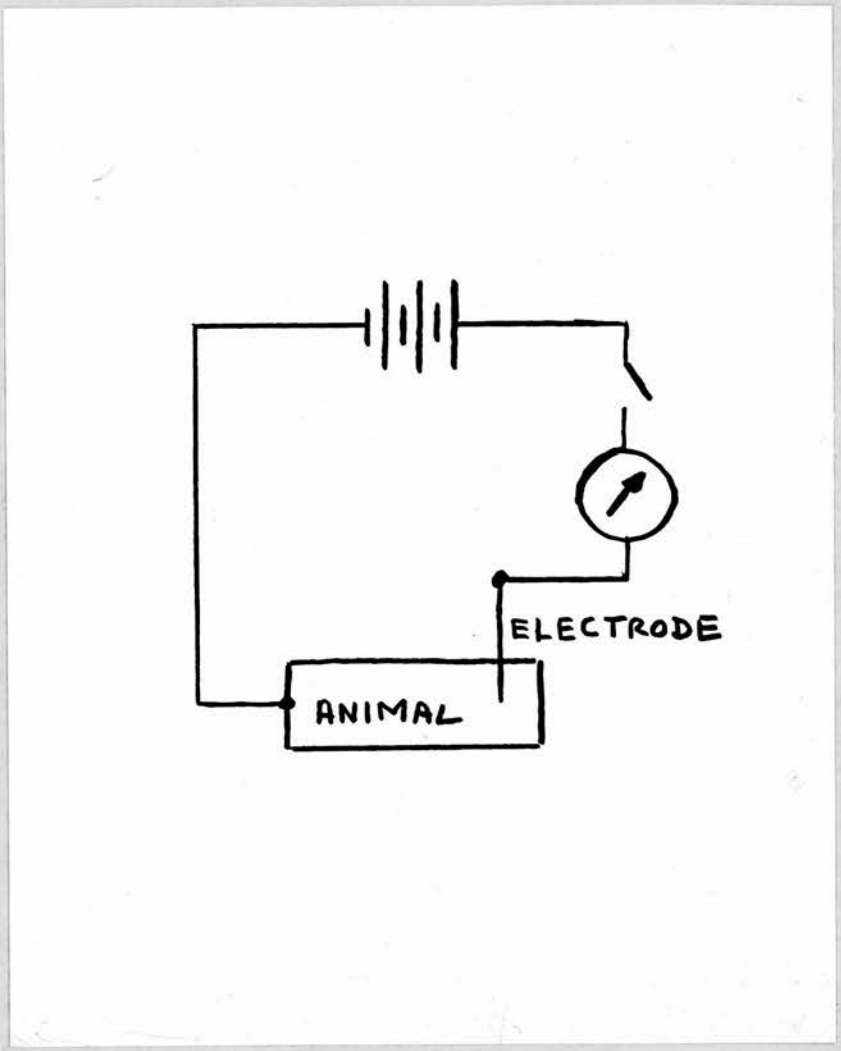

FIG.29 Electrical circuit used for causing an electrolytic lesion of the locus coeruleus.

RESULTS

Effect of anaesthesia and surgery on metabolism

Since most of the stimulation and ablation experiments involved anaesthetising the animals, it was necessary to test whether the anaesthetic had any effect on the level of MHPG in the brain. Diaz (1968) found that diethyl-ether anaesthesia produced a large rise in the 5HT metabolite 5-hydroxyindole acetic acid (5HIAA), and Shields (1972) found that urethane doubled the 5HIAA level in rat brain, whilst halothane had no effect. It was also possible that there might be some effect on metabolism as a result of the surgical procedures.

Four rats, therefore were anaesthetised with halothane in oxygen, an electrode implanted in the region of the locus coeruleus, and these animals left for 1 hour without stimulation and then killed. The concentration of MHPG was then measured in cerebral cortex and the values compared to a group of three rats of the same litter which received no treatment, the results in table 8 showing that the procedure had no effect on the level of the noradrenaline metabolite in cortex.

The effect of electrical stimulation of the locus coeruleus on the concentration of MHPG in rat brain

a) Confirmation of electrode position

In a preliminary series of experiments, the position of the stimulating electrode in relation to the locus coeruleus was determined by taking frozen serial sections

TABLE 8 The effect of anaesthesia on the endogenous concentration of total HMPG (free + conjugated) in rat cerebral cortex.

Non-Experimental		Halothane in Oxygen		
Left (ng/g)	Right (ng/g)	Left (ng/g)	Right (ng/g)	
94	117	74	91	
78	61	31	119	
130	81	125	105	
		102	119	
101 ± 27	86 ± 28	83 ± 40	109 ± 13	Mean ± SD
L + R,	94 ± 26	L + R,	96 ± 31	Mean ± SD

of the brain stem and cerebellum and staining with toluidine blue. Fig.30 shows part of the electrode track ending just dorsal to the locus coeruleus. When the electrode is withdrawn from the brain, the track fills up with blood, which forms brown crystals during the histological procedure. The locus coeruleus (LC) is the compact nucleus made up of small cell bodies. The adjacent group of larger cells forms part of the nucleus of the mesencephalic tract of the trigeminal nerve.

In subsequent stimulation experiments, routine histology was not carried out, therefore no selection of animals was made on this basis.

b) The effect on HMPG concentration

The effect of stimulating the left nucleus locus coeruleus on the concentration of free HMPG and HMPG-SO_4 measured separately in whole rat brain is shown in Fig.31. Stimulation caused a significant ($P < 0.05$) 60% increase in conjugated HMPG but had no effect on the level of the free glycol. Control values were obtained from animals with an implanted electrode but not stimulated.

Following on from the work of Ungerstedt (1971) who showed that there was a direct, largely uncrossed pathway from the locus coeruleus to the cerebral cortex, it was decided to investigate whether electrical stimulation of this nucleus would cause an increase of HMPG in cortex, as a result of an increased release of noradrenaline. In the following experiments, therefore, the left locus coeruleus of a group of rats was stimulated electrically,

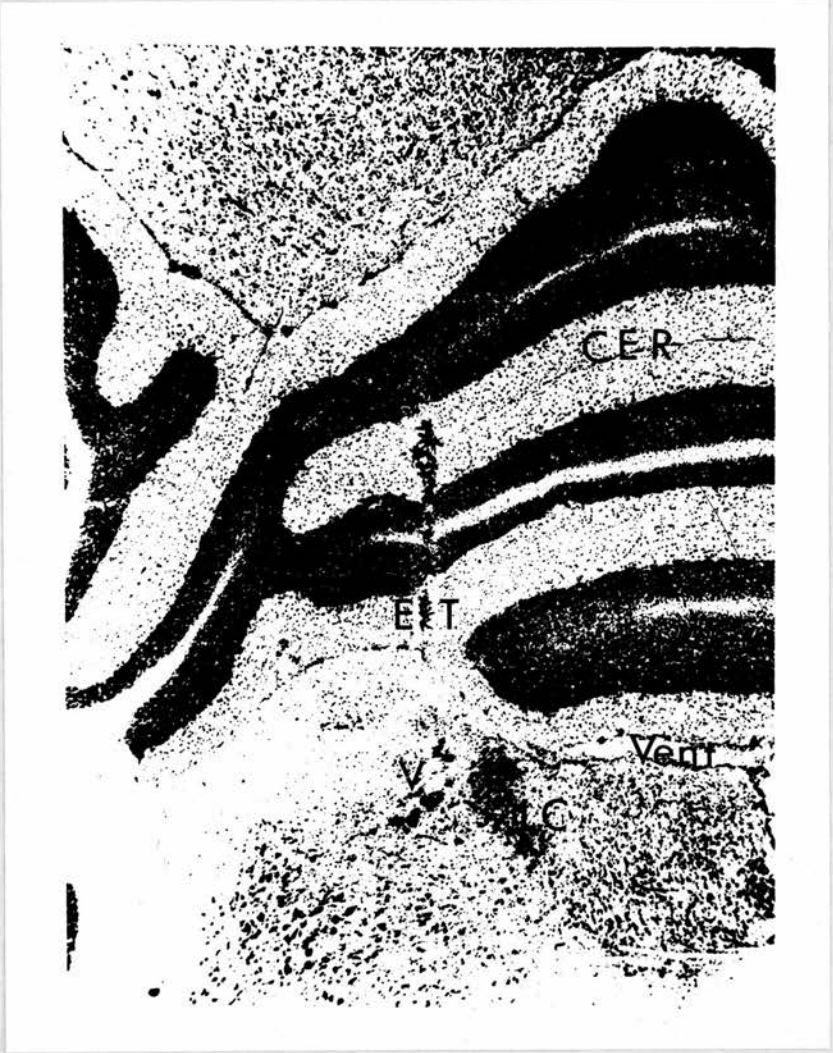

FIG. 30 Plate of frontal section of rat brain at the level of the 4th ventricle, showing the electrode track ending just dorsal to the left locus coeruleus. Toluidine blue stain X30.

Key
CER = cerebellum
ET = electrode track
LC = locus coeruleus
V = nucleus of mesencephalic tract of trigeminal nerve
Vent = 4th ventricle.

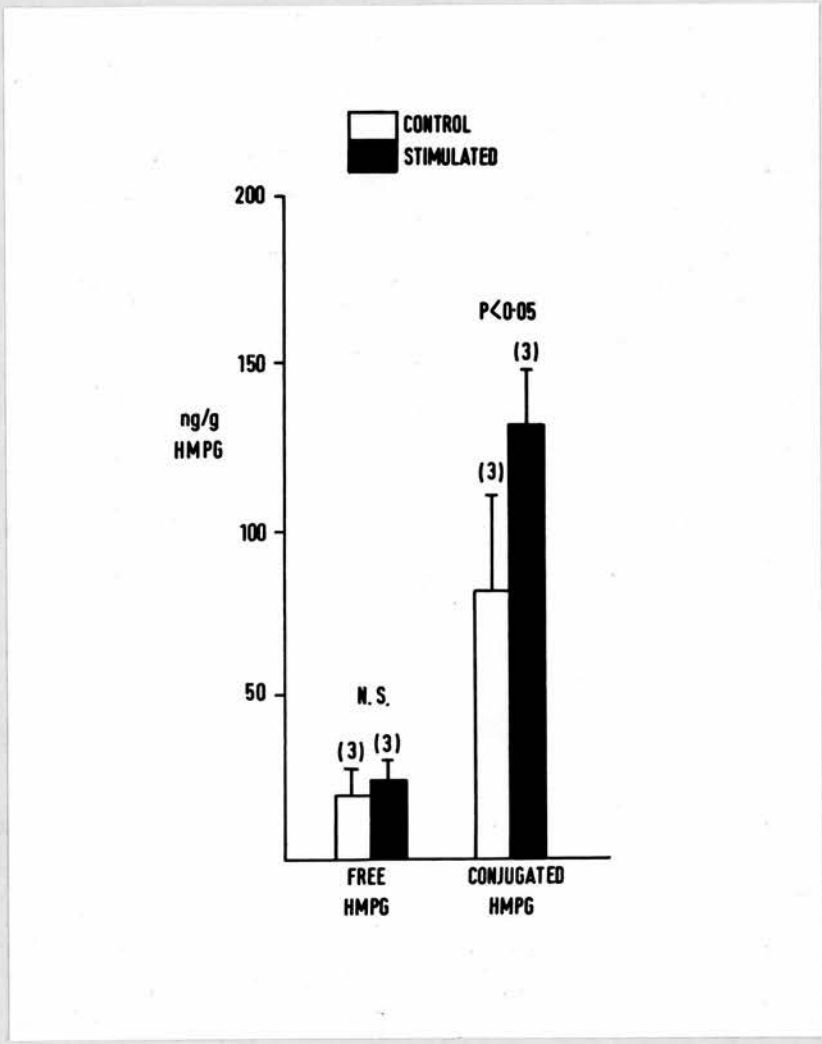

FIG. 31 The effect of electrical stimulation of the locus coeruleus on the concentrations of free and conjugated HMPG in whole rat brain. Comparison was made with animals in which an electrode was implanted but not stimulated. Mean ± SD (number of animals) P-values calculated from Students t-test.

and the separate left and right cerebral cortex taken and analysed for the concentration of total HMPG (free + conjugated). Control groups had electrodes implanted but were not stimulated. Fig. 32 shows that stimulation did result in an increase of the noradrenaline metabolite in cortex, but, although there was a trend for the cortex on the stimulated side to have greater values than the contralateral cortex, there was no significant difference between the two sides. It was felt, however, that the possible unilateral nature of the rise of HMPG should be investigated. Two modifications to the technique were therefore made. Firstly, unipolar electrodes were used in place of bipolar ones, in an attempt to localise the stimulus, since it was thought that the reason for the bilateral rise might be due to simultaneous stimulation of the opposite nucleus locus coeruleus, and secondly, the stimulus current strength was altered for the same reason.

Tables 9 and 10 show the effect of the type of electrode on the level of total HMPG in cerebral cortex using a stimulus current strength of 0.2 mA (table 9) and 0.05 mA (table 10). Control values, which ranged between 86 ng/g and 120 ng/g were normal and for the sake of clarity are not included in the tables. It can be seen that with a current strength of 0.2 mA, stimulation with both unipolar and bipolar electrodes resulted in a similar rise of metabolite on both sides of cortex. At 0.05 mA, however, examination of the individual results shows that with unipolar electrodes, two animals had a rise of HMPG in the

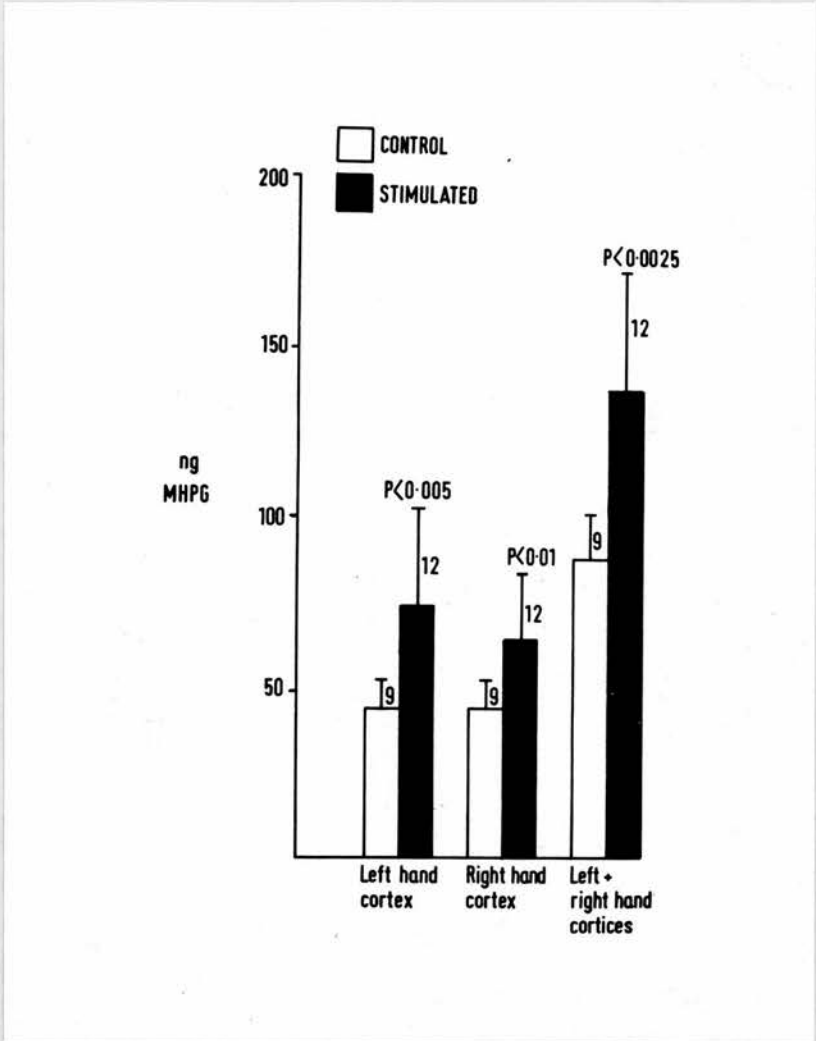

FIG.32 The effect of electrical stimulation of the left locus coeruleus on the concentration of total HMPG (free + conjugated) in rat cerebral cortex. P-values are calculated using Students t-test.

TABLE 9 The effect of the type of electrode on the concentration of total HMPG (free + conjugated) in rat cerebral cortex following stimulation for 45 min using 0.2 mA constant current pulses at 10 per second.

Unipolar		Concentric Bipolar		
Left (ng/g)	Right (ng/g)	Left (ng/g)	Right (ng/g)	
233	247	163	146	
188	230	170	175	
215	248	196	206	
312	283	138	126	
291	243	218	197	
248 ± 52	250 ± 19	177 ± 28	170 ± 30	Mean ± SD

TABLE 10 The effect of the type of electrode on the concentration of total HMPG (free + conjugated) in rat cerebral cortex following stimulation for 45 min using 0.05 mA constant current pulses at 10 per second.

Unipolar		Concentric Bipolar		
Left (ng/g)	Right (ng/g)	Left (ng/g)	Right (ng/g)	
107	102	342	332	
118	109	300	377	
136	102	371	305	
177	109	359	284	
230	155			
154 ± 50	115 ± 22	343 ± 27	324 ± 35	Mean ± SD

cortex of the stimulated side with control values on the contralateral side, one animal showed increases on both sides but with a greater increase on the stimulated side and two animals had control values (presumably because of incorrect electrode placement). Using concentric bipolar electrodes at this current strength, values were similar on both sides of cortex.

When the current strength was increased to 0.1 mA (table 11) there was a greater increase in the levelof HMPG in the cortex on the stimulated side than at 0.05 mA, but also a slight increase in the level of the metabolite on the contralateral side. Individual results show that three rats of the five had a marked differential between stimulated and contralateral cortical concentrations of HMPG, one rat had similar raised values for both sides and one rat had control values. These results are summarised in Fig. 33. Using a paired t-test analysis with all values included, there was a significant difference between stimulated and contralateral sides at current strengths of 0.05 mA ($P < 0.05$) and 0.1 mA ($P < 0.025$).

An investigation of the optimum frequency of stimulation using 0.1 mA constant current pulses 2 msec in duration showed that (Fig. 34) the greatest mean percentage increase of total HMPG (free + conjugated) occurred at a frequency of 2 per second (P 0.0005) which is near the spontaneous firing rate of the cells of the locus coeruleus reported by Graham and Aghajanian (1972). Stimulation at a frequency of 5 Hz 10 Hz and 20 Hz , however, also

TABLE 11 The effect of stimulating the left locus coeruleus for 45 min on the concentration of total HMPG (free + conjugated) in rat cerebral cortex, using unipolar electrodes with 0.1 mA constant current pulses at 10 per second.

Stimulation at 0.1 mA	
Left (ng/g)	Right (ng/g)
262	144
280	175
275	156
293	261
121	118
246 ± 71	171 ± 54

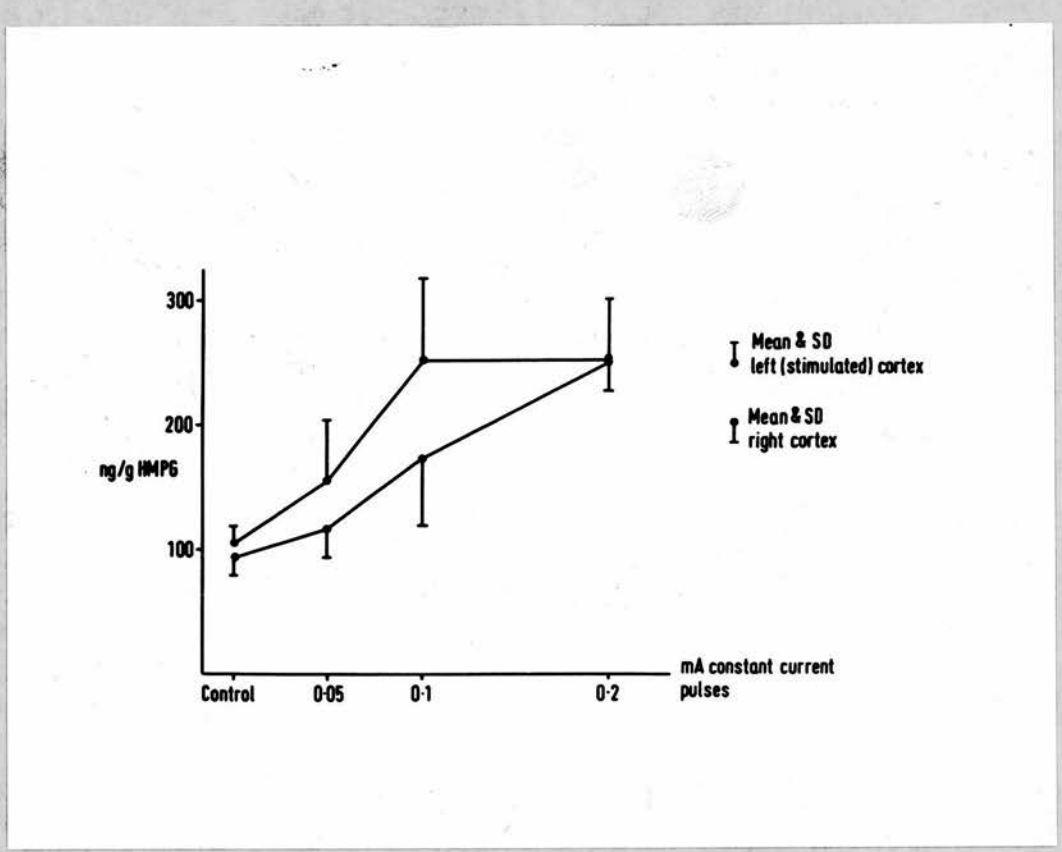

FIG. 33 The effect of varying the stimulus current strength on the mean ± SD concentration of total HMPG (free + conjugated) in left (stimulated) and right (unstimulated) cortex. N = 5 for each point. Paired t-test analysis at the various current strengths showed a significant difference at 0.05 mA ($P < 0.05$) and 0.1 mA ($P < 0.025$).

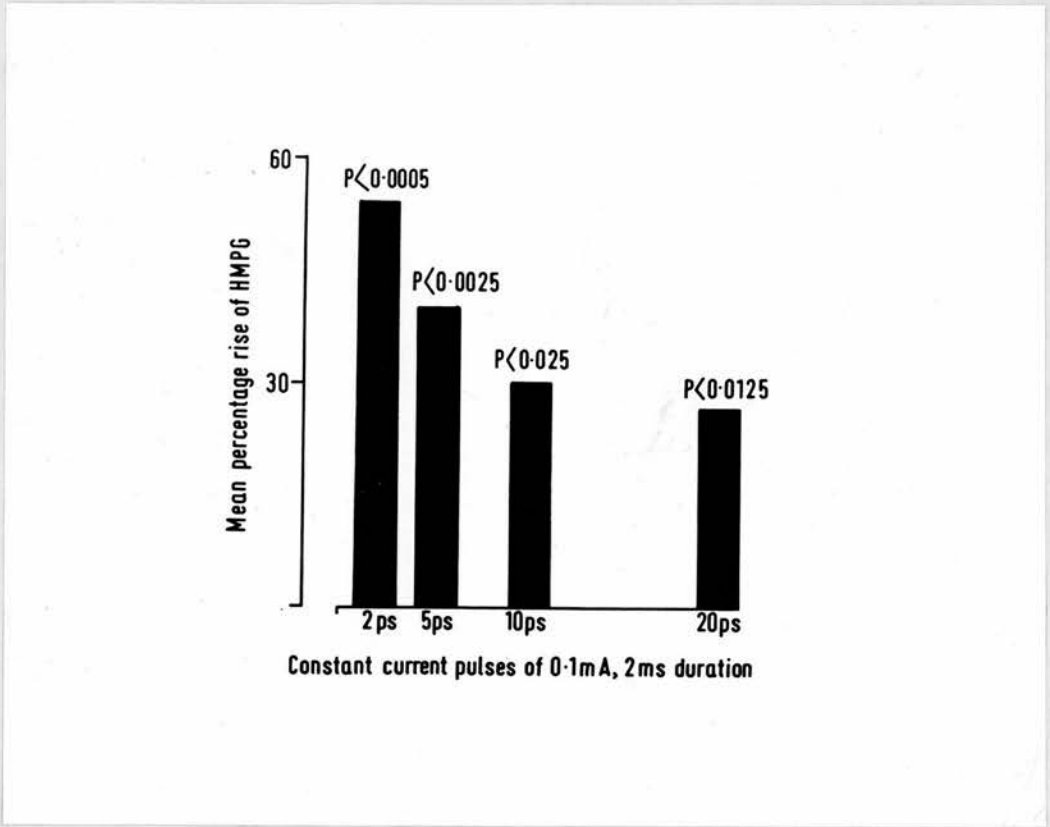

FIG.34 The effect of varying the frequency of stimuli on the rise of total HMPG in rat cerebral cortex, showing the mean percentage rise (n = 4 for each stimulated group and for controls). Single tailed P-values are calculated using the Students t-test are related to the mean control value.

caused a significant rise in the noradrenaline metabolite (5 Hz , $P < 0.0025$; 10 Hz , $P < 0.025$; 20 Hz , $P < 0.0125$; Student t-test).

A preliminary experiment was carried out to assess the effect of electrical stimulation on the relative concentrations of DHPG and HMPG in rat cerebral cortex. The left locus coeruleus of a group of five rats was stimulated for 45 min using 0.2 mA constant current pulses at 10 per second. A control group had electrodes implanted but was not stimulated. Pooled left and right cortex was analysed for the concentration of HMPG and DHPG using the modified method described in Section I. Examination of the individual figures in Table 12 shows that two of the five animals stimulated had raised levels of both HMPG and DHPG in cerebral cortex and the other three animals had levels which were of the same order as control values. Although the mean percentage differenceof HMPG and DHPG was not significantly different between the stimulated and control groups, the difference in the two metabolites was much less between the two rats with raised levels and the other three rats of the same group.

<u>The relation between electrical self stimulation behaviour in animals with electrode tips in the region of the locus coeruleus, and the cerebral cortex HMPG concentration</u>

Crow et al (1972) have shown that the positive reinforcing behavioural phenomenum of electrical self stimulation in the region of the fourth ventricle, is

TABLE 12 The effect of electrical stimulation of the locus coeruleus for 45 min on the concentrations of total (free + conjugated) HMPG and DHPG in rat cerebral cortex, showing that where stimulation caused an increase of HMPG (Stimulated 2 and 3) there was also an increase of DHPG.

		HMPG (ng/g)	DHPG (ng/g)	Percentage Difference DHPG = 100%
Control	1	142	90	58
	2	153	106	44
	3	163	136	20
Mean ± SD		153 ± 9	111 ± 19	41 ± 16
Stimulated	1	172	93	85
	2	211	172	23
	3	196	189	4
	4	162	93	74
	5	170	92	86
Mean ± SD		182 ± 18	128 ± 43	54 ± 34

closely correlated with the site of the electrode tip being close to the locus coeruleus and the nucleus of the mesencephalic tract of the trigeminal nerve. Since electrical stimulation of the locus coeruleus under halothane anaesthesia causes an increase in the level of HMPG in rat cerebral cortex, a similar increase may occur during conscious electrical self-stimulation. Experiments were, therefore, designed to test this.

Two control groups of animals were used, a non-experimental group and a group which had electrodes implanted but were not stimulated. There was no difference between the cortical HMPG concentration of the two groups, and so the results from the two were pooled, giving a mean cortical concentration of total HMPG (free + conjugated) of 146 $\pm$ 38 ng/g (14) (mean $\pm$ SD, number of determinations) for Hooded Lister rats.

Animals showing a positive self stimulating response were also divided into two groups. Six rats were allowed to consciously self stimulate for one hour, receiving a 200 msec train of 0.5 msec pulses at 100 H_z each time the lever was pressed (an average of about once every 4 seconds). A further three rats were anaesthetised with halothane in oxygen and operator stimulated at a comparable rate, that is once every 4 seconds). The current strength varied for each rat, predetermined in the conditioning period. As a further control, a group of 5 animals, which had electrodes implanted and had received operant conditioning

but had not shown self stimulating behaviour, were anaesthetised with halothane in oxygen and operator stimulated at the same rate as the self stimulation group.

After one hour, the rats were killed by cervical fracture, the brains removed and the cortex dissected for HMPG analysis. The brainstem and cerebellum was removed and placed in 10% Formal-saline prior to histological analysis of the electrode tip position using the Kluver-Barrera technique.

The concentration of total HMPG (free + conjugated) in left and right cerebral cortex is summarised in table 13. All animals had stimulating electrodes implanted in the left locus coeruleus. In the group of five rats which had been trained, but had not shown self stimulating behaviour, after one hours electrical stimulation under halothane anaesthesia the cortical concentration of HMPG was identical with the control group. The group of three self stimulating animals which were operator stimulated under halothane anaesthesia, however, showed a marked ipsilateral rise of HMPG. The left (stimulated) cortex contained more than double the control value of the metabolite, whilst the contralateral side showed only a smaller increase in HMPG. The group of six positive animals allowed to consciously self stimulate for one hour showed a comparable total increase in HMPG but with a less marked ipsilateral nature. Table 14 shows the response rate, stimulation current strength and HMPG concentration for each positive animal. No correlation was

TABLE 13 Mean ± SD concentration of total HMPG (free + conjugated) in left and right cerebral cortex. All animals had stimulating electrodes implanted in the left locus coeruleus. P-values are from single tailed Students t-test and refer to the control values. The number in brackets is the number of animals.

* $P < 0.01$

** $P < 0.0005$

Procedure	Type of Experimental rat	ng/g total HMPG	
		Left Cortex	Right Cortex
Unstimulated Controls		151 ± 38(7)	141 ± 36(7)
Operator-Stimulated Under Halothane Anaesthesia	Non Self-Stimulating	159 ± 28(5)	149 ± 15(5)
	Self-Stimulating	328 ± 56**(3)	175 ± 30(3)
Self-Stimulation In Conscious State	Self-Stimulating	251 ± 21**(6)	206*± 40(6)

TABLE 14 The relation between the stimulus current strength, response rate and cortical HMPG concentration in self stimulating rats.

Rat Number	Current Strength μA	Response or Stimulation Rate per Hour	HMPG Concentration ng/g	
			Ipsilateral	Contralateral
Conscious Self Stimulation				
1	250	696	240	152
2	150	1500	244	227
3	150	821	286	164
4	200	1028	220	242
5	150	650	267	192
6	250	750	251	260
Mean ± SD	192 ± 45	907 ± 291	251 ± 21	206 ± 40
Stimulation Under Halothane				
1	600	900	339	216
2	200	900	322	144
3	300	900	263	164
Mean ± SD	366 ± 170	900	308 ± 33	175 ± 30

found between either stimulating parameters and the HMPG concentration.

These results were then correlated with the histological analysis of the position of the electrode tip in relation to the nucleus locus coeruleus. Two observers made independent assessment of the electrode tip position without knowledge of which group the animal was part. Fig.35 shows diagrams drawn from frontal sections of rat brainstem and cerebellum around the region of the fourth ventricle. The positions of the electrode tips in relation to the locus coeruleus are shown by the square markers (negative animals) and the circular markers (positive animals). It can be seen, as shown previously by Crow et al (1972) that the electrode tip position from all positive self stimulating animals were clustered at the locus coeruleus, and the negative animals had tip positions outside the area.

<u>The effect of electrolytic ablation of the nucleus locus coeruleus on the cortical concentration of total HMPG</u>

Since electrical stimulation causes an increase in the major metabolite of noradrenaline, presumably through an increase in the turnover of the catecholamine at the terminals in cortex, it was thought reasonable that destruction of this system by electrolytic ablation of the nucleus locus coeruleus might cause a reduction of the metabolite in the cerebral cortex. Experiments were therefore designed to test this.

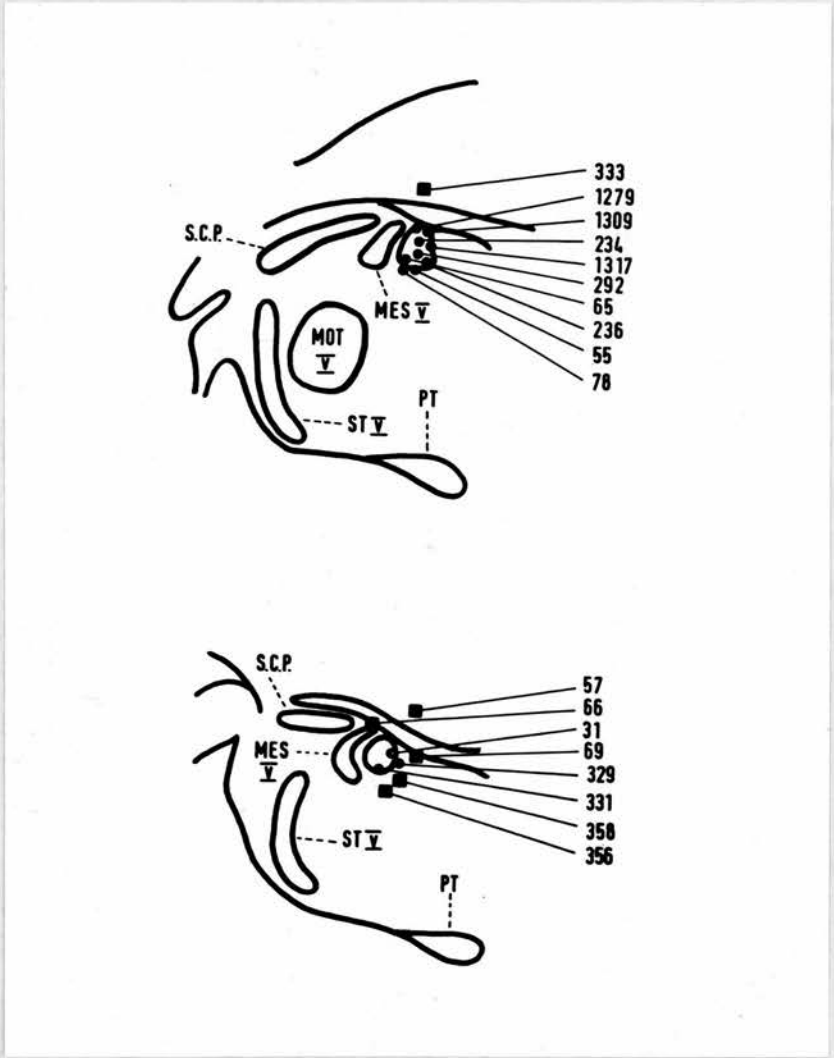

FIG. 35 Diagrams drawn from plates of rat brain stem and cerebellum at the level of the 4th ventricle, showing the position of the tips of electrodes with respect to the locus coeruleus. Filled-in circles represent tip positions of animals showing self stimulating behaviour, and the filled-in squares, the tip positions of animals not showing self stimulating behaviour.

Eighteen female albino Wistar rats were divided into three groups of six rats. In one group the left locus coeruleus was electrolytically ablated, in another group both nuclei were ablated and in a further control group, burr holes were drilled in the skull bilaterally, the dura removed, but no electrode implanted. After three weeks, to allow for the degeneration of the pathway, the animals were killed, the brainstem and cerebellum removed for histological examination and the cortical level of HMPG measured as before.

Table 15 shows that there was a significant reduction in the total HMPG (free + conjugated) concentration on the side of the brainstem lesion in unilaterally ablated rats, and a similar fall in the HMPG concentration in both cortices of the bilaterally ablated rats.

The extent of the lesion damage following electrolytic ablation of the locus coeruleus is shown in Fig.36 (unilateral ablation) and Fig.37 (bilateral ablation). A charge of 6 millicoulombs produced an area of damage at the electrode tip equivalent to a sphere between 0.5 mm and 0.8 mm in diameter.

Table 16 shows the relation between the lesion damage to the locus coeruleus and the HMPG concentration in the cerebral cortex. In four animals of the unilateral group there was a histologically confirmed lesion or partial lesion of the locus coeruleus. This was correlated with a significant ($P < 0.0025$) reduction in the HMPG concentration on the same side as the lesion when compared to the

TABLE 15 Mean ± SD concentration of total HMPG (free + conjugated in rat cerebral cortex following electrolytic ablation of one or both locus coeruleus. P-values are calculated from Students t-test and refer to control values.

* $P < 0.025$

** $P < 0.01$

*** $P < 0.005$

	N	Left Cortex (ng/g)	Right Cortex (ng/g)
Control	6	103 ± 33	96 ± 26
Unilateral (Left) Lesion	6	40 ± 19***	106 ± 23
Bilateral Lesion	4	45 ± 20*	30 ± 6**

FIG. 36 Plate of frontal section of rat brain at the level of the 4th ventricle showing the appearance of a unilateral ablation of the locus coeruleus(LC). Toluidine blue stain X30.

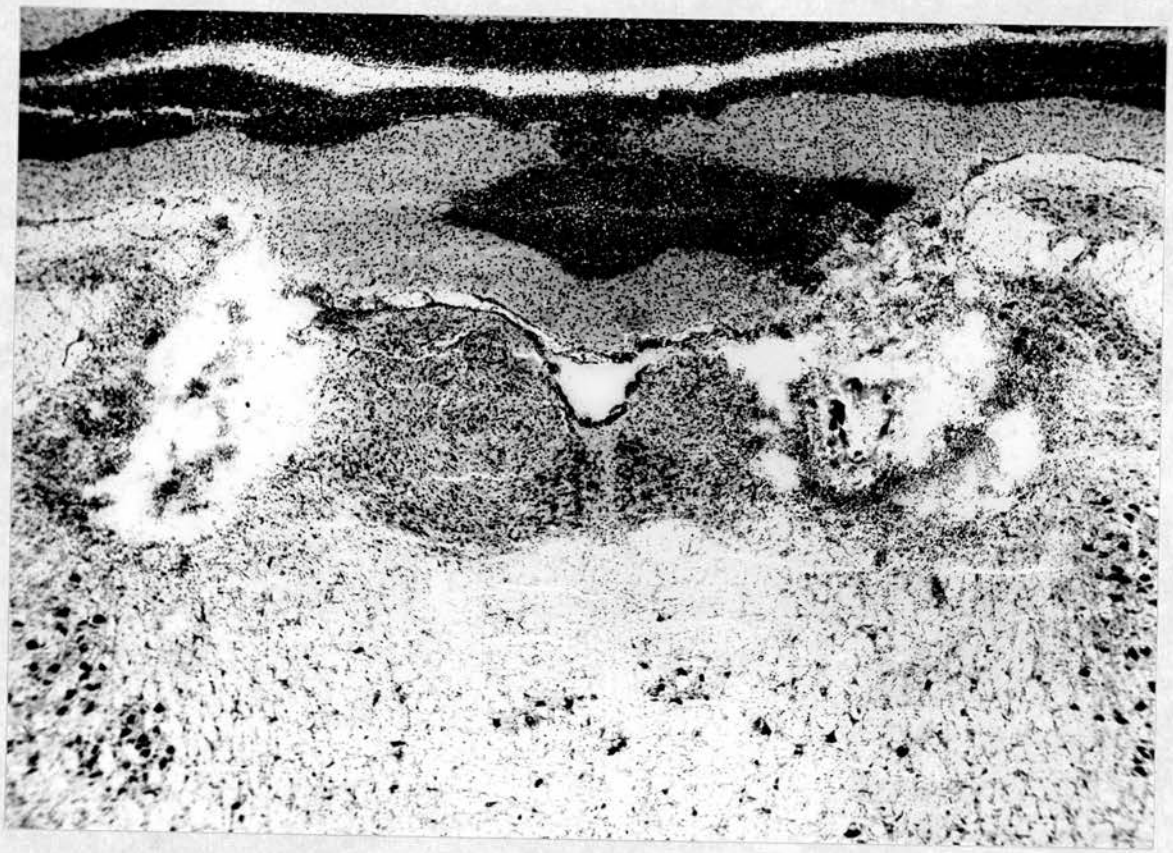

FIG. 37 Plate of frontal section of rat brain at the level of the 4th ventricle, showing the appearance of a bilateral ablation of the locus coeruleus. Toluidine stain X30.

TABLE 16 The relation between histologically confirmed lesion damage to the locus coeruleus and the concentration of total HMPG (free + conjugated) in rat cerebral cortex.

Key
UL - unilateral (left sided) lesion
BL - bilateral lesion
CC - control

Lesions
2 - intact
1 - partially ablated
0 - completely ablated.

* These animals selected on the basis of histology as a group show a $P < 0.0025$ significant fall in HMPG concentration on the side of the lesion, using a paired t-test.

	Rat	Locus Coeruleus L	Locus Coeruleus R	Cortical HMPG ng/g L	Cortical HMPG ng/g R
*	UL1	0	2	30	82
*	UL2	0	2	23	105
*	UL3	1	2	23	105
	UL4	2	2	66	75
*	UL5	1	2	66	127
	UL6	-	-	30	141
	CC1	2	2	171	57
	CC2	2	2	118	127
	CC3	2	2	82	75
	CC4	2	2	89	114
	CC5	2	2	75	82
	CC6	2	2	82	118
	BL1	0	1	23	43
	BL2	0	1	75	43
	BL3	1	1	52	-
	BL4	1	1	30	30

values for the contralateral cortex using a paired t-test analysis.

Fig.38 shows the scatter of the results when the histological appearance of the locus coeruleus is described as intact, partially ablated, or completely ablated. It was interesting to note that a complete absence of metabolite was never noticed in these experiments.

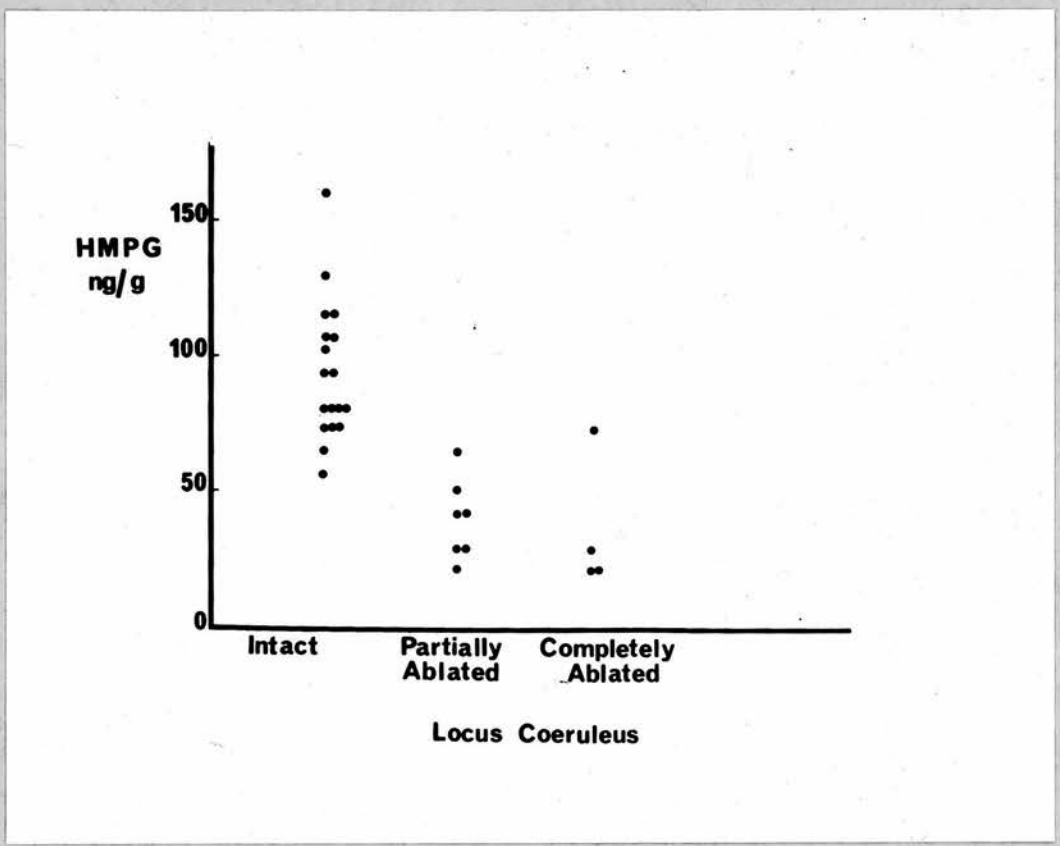

FIG. 38 Scatter diagram showing the relation between HMPG concentration in rat cerebral cortex and the state of the locus coeruleus following ablation experiments.

DISCUSSION

The stereotaxic placement of an electrode for stimulation or ablation can theoretically be reproducibly placed within a cube with the dimensions of a tenth of a millimeter, which for most structures in rat brain would be adequate. This accuracy depends on a) that every rat of the same weight has the same skull shape and size, b) that during the development of the brain the various structures are laid down with a uniform pattern and c) that the experimenter can secure the rat in a constant orientation.

The use of a single strain of rats, all within a close weight range, with a good stereotaxic frame minimizes the errors, but even so, the chance of correctly placing the electrode tip at a structure as small as the nucleus locus coeruleus is much less than 100%. In a separate series of experiments, making unilateral lesions of the locus coeruleus and measuring the production of (^{14}C) cyclic AMP from (^{14}C) adenine in cortical slices from these animals, 16 of the rats had lesions in the locus coeruleus and in 8 rats the lesion completely missed the locus coeruleus, a success rate of 66%.(Eccleston 1973). Using the co-ordinates of Crow et al (1972) who used the lambda suture apex position as an (AP) co-ordinate zero, the success was much less than this, presumably because of the non-uniformity of the rat skull markings.

Toluidine blue staining combined with the cryostat method of sectioning brain tissue provides a very rapid

method for analysing rat brain, with results available within 90 min of killing the animal, but the method is limited in the amount of information it can supply since toluidine blue stains only cell nuclei. The nucleus locus coeruleus, is, however, a compact and discrete nucleus and stains up well (Fig.30). This technique is therefore useful for determining the integrity of the nucleus following electrolytic ablation. The Klüver-Barrera technique is, however, more useful for determining the electrode tip position since with this method the collection of every section is guaranteed, and the double staining using Luxol fast blue and cresyl violet reveals more details of structure.

Three types of electrodes were used to stimulate the locus coeruleus. The concentric bipolar electrodes, although well suited for the stimulation of the raphe system, were too large (0.5 mm) and the volume of tissue stimulated too great to ensure that only one of the two nuclei loci coerulei was stimulated. Using the unipolar electrodes, (0.3 mm thick) more unilateral control of stimulation was possible, but a bilateral rise of HMPG was seen in many cases. The third type of electrode used to stimulate the locus coeruleus was the twisted bipolar type used for electrical self stimulation. These electrodes by their design had a very limited sphere of action, a fact supported by the histology, since electrode placements just outside the locus coeruleus did not support self stimulation and did not give rise to an increase in the NA metabolite

during operator stimulation under halothane anaesthesia. Even using these very specific electrodes with a localised sphere of action, in five of the nine positive animals tested there was a substantial rise of HMPG in the contralateral cortex along with an increase in metabolite on the stimulated side, whilst the remaining four showed a unilateral response to stimulation.

From these results, then, it would appear that current spread to the opposite locus coeruleus was not the factor which caused the contralateral effect. The most obvious alternative is that there is a pathway arising from the locus coeruleus which innervates the contralateral cortex. This, however, is not supported by the results of lesion experiments. Ungerstedt (1972) has shown that unilateral lesions of the locus coeruleus cause a depletion of catecholamine fluorescence in the homolateral cortex, which fact is consistent with the effect of locus coeruleus ablation on HMPG levels reported here, where a unilateral lesion caused a marked reduction in the metabolite on the side of the lesion without affecting the contralateral cortex. Similar results have been obtained by Korf et al (1973) for HMPG-SO_4, and they showed that the reduction in the noradrenaline concentration was also confined to the side of the lesion. If there was a pathway from the LC to the contralateral cortex, one would expect to see a reduction in the metabolite on both sides of cortex although one might predict a smaller reduction on the opposite side.

Although Ungerstedt describes the pathway to the cerebral cortex as uncrossed, he does indicate that some axons from the locus coeruleus cross the mid line medial to the nucleus, and suggests that these axons probably ascend to other brain areas, since Loizou (1969) had shown that bilateral lesions of the locus coeruleus are more effective in denervating the hypothalamus, than unilateral lesions. Olson and Fuxe (1972) have investigated further this medial pathway in their studies of the cerebellar innervation from thelocus coeruleus. The medial fibre projection apparent in horizontal sections, seems to go to the raphe system and they suggest it also partly represents crossing fibres. It appears to originate from the most ventromedial part of the locus coeruleus which has the largest cell bodies and shows the strongest fluorescence intensity. No bundle formation was observed by these authors, which suggests that the function of the medial pathway is to innervate the raphe system, and/or that the fibres pass through the raphe system and innervate the opposite locus coeruleus. If the latter was true, the medial pathway might then be acting as a 'servo mechanism', recruiting the actions of the opposite nucleus locus coeruleus. Such a mechanism would be consistent with the lesion results since removal of the LC on one side should not necessarily affect the efficiency of the intact side but only obviate its 'recruitment'. Also, a truly unilateral response during stimulation of the LC might only occur providing the cells of the medial pathway were not activated by the stimulating current,

and conversely, a contralateral effect would only be noticed when this system was activated.

The activation of the medial pathway would depend on two interrelated variables, the stimulus current strength and the position of the electrode tip. Using unipolar electrodes, a stimulating current strength of 0.2 mA always gave rise to a bilateral effect whereas current strengths of 0.1 mA and 0.05 mA produced a distinct unilateral rise of HMPG in some animals. Using the concentric bipolar electrodes, however, a bilateral effect was obtained at all current strengths used. In the self stimulation experiments, the current varied from 0.15 mA to 0.6 mA depending on the animal. There was, however, in these experiments, no relation between current strength and the nature of the rise of HMPG in cerebral cortex.

One other group has produced results showing the effect of stimulation and electrolytic ablation of the locus coeruleus on noradrenaline metabolism in rat cerebral cortex. Korf et al (1973) used the fluorimetric method of Meek and Neff (1972) to determine HMPG-SO_4 following its isolation on a column of DEAE-Sephadex. Their methods were very similar to the ones reported here, which made the results easier to compare. Their main difference in technique was in the method of stimulation, where they used monophasic pulses of 2 msec duration at a current strength of 0.5 mA. This may account for the difference they obtained for the optimum frequency of stimulation. They found that when the locus coeruleus was stimulated for

30 min, there was a linear rise in HMPG-SO_4 as a function of the frequency, reaching a plateau at a frequency of 20 H_z, whereas the results here show that 45 min stimulation using 0.1 mA constant current pulses alternating in polarity and at 2 Hz, gave the largest rise of total HMPG in cortex with less of an effect at higher frequencies (Fig.34). In their optimum frequency experiment, they express the rise of HMPG-SO_4 on the stimulated side as a function of the level on the non-stimulated side, the latter which they made 100%. They do not indicate whether they noticed a contralateral rise of metabolite, although using this method to express their results suggests that this may have been the case. Neither do the authors comment on the unilateral rise of HMPG-SO_4, although they do suggest that their lesion results are in agreement with the work of Ungerstedt (1971) that there is no significant mid line crossing of noradrenaline pathways from the LC to the contralateral cortex and hippocampus, since there were no changes in noradrenaline and HMPG-SO_4 on the side opposite the lesion.

Their results that the HMPG-SO_4 level in cerebral cortex rises on stimulation are consistent with the results presented here. They further suggest that since after an initial rise of HMPG-SO_4 the concentration levels off at a new steady state, this could be explained by assuming an extremely efficient transport of HMPG-SO_4 out of brain, or by a decreased formation of HMPG-SO_4 after about 15 min of stimulation. The former idea is in agreement with the

idea of Meek and Neff (1972) who showed that the transport of both VMA and HMPG-SO_4 from brain was by a 'probenecid sensitive' acid transport system. This mechanism was blocked by probenecid, causing a dose dependent increase in the concentration of HMPG-SO_4 in rat brain, 90 min after the injection. Attempts to reproduce this, however, measuring HMPG and HMPG-SO_4 by gas chromatography have not been successful. No change in either metabolite was noticed between rats 2 hours after an injection of 200 mg/kg of probenecid interperitoneally and control rats (unpublished observations).

In a preliminary experiment, measuring DHPG and HMPG in the same sample, using the modified technique described in the first section, it was seen that, after stimulating the left locus coeruleus, where there was a rise of HMPG in cortex, a similar rise of the dihydroxyphenyl derivative also occurred (Table 12), thus indicating further that the contribution of noradrenaline metabolised to DHPG is probably greater than has previously been suggested.

Work on the mechanisms of sleep has shown that the two states, slow wave sleep (SWS) and rapid eye movement sleep (REM sleep) (or paradoxical sleep) are probably regulated in some way by monoaminergic systems having their nerve cellsin the brainstem, since lesions of specific nuclei in this area cause alterations in the electrical activity usually associated with sleep (Jouvet 1966). Thus,

in cats destruction of a large part of the raphe nuclei induces total insomnia in the first few days, although some recovery of SWS then occurs, whereas a return of REM sleep in these cats never occurs. Pharmacological depletion of 5HT using parachlorophenylalanine gives similar results, causing a gradual decrease in both states of sleep after a delay of about twelve hours leading eventually to total insomnia (Mouret 1967).

Following bilateral lesions of the locus coeruleus there is a good correlation between the extent of LC damage and the fall in cerebral NA concentration and this is coupled with a decrease in REM sleep without altering the amount of SWS or the cerebral concentration of 5HT (Jouvet 1969). This decrease in REM sleep is apparent as a striking behavioural syndrome in the cats 'in which the cat stands up and exhibits anger or fear and may attack imaginary enemies, moving its head around for one or two minutes. The ocular behaviour of these animals is, however, that of deep sleep and they do not react to visual stimuli'. Control lesions placed around the locus coeruleus did not produce this effect, except for two animals which had lesions medial to the LC, (Jouvet 1966). In view of the work of Olson and Fuxe (1972) who described a medial pathway from the LC to the raphe (or passing through it), these latter results are interesting since the raphe nucleus is strongly implicated in both states of sleep. The idea that the locus coeruleus might then be involved in the initiation of REM sleep is hardly consistent

with observations of the behaviour of rats undergoing conscious self stimulation, which activity does not induce sleep, but rather increases the arousal of the animal (Crow et al 1972). More recent work performed with cats to elucidate the role of the locus coeruleus in sleep and waking behaviour has also thrown a contradictory light on the lesion experiments. Chu and Bloom (1973) have shown that the unit activity of LC neurones in the 'quiet waking state and in SWS is at a low frequency, about 4.5 and 4.2 Hz respectively, whereas in REM sleep and the 'attentive waking state' the rate is more than double this, about 10.0 and 11.6 Hz respectively, showing that the increased cortical activity which occurs in the attentive waking state and is seen to occur in REM sleep (by the EEG activity) is correlated with an increased LC firing rate, a fact more consistent with ideas implicating the LC in the maintenance of the arousal of the animal (Crow 1973).

It is now almost certain that electrical self stimulation behaviour in rats is correlated with the electrode tips being at the cell bodies or axonal pathways of dopamine containing or noradrenaline containing systems in brain (Crow 1972; Crow et al 1972). Previous extensive mapping of brain showing sites supporting self stimulation (Olds and Milner, 1954; Olds and Olds, 1963; Wilkinson and Peele, 1963; O'Donohue and Hagamen, 1967; Routtenberg and Malsbury, 1969) coincide well with the areas through which the axonal projections of the catecholamine systems pass, areas such as the medial forebrain bundle, the mid brain

tegmentum and the superior cerebellar peduncle. Results presented here support the catecholamine hypothesis since only electrodes with tips at the locus coeruleus gave an increase of HMPG during conscious electrical self stimulation and conversely, animals which did not show self stimulation behaviour did not give rise to an increase of the NA metabolite even after 1 hour of operator stimulation at the same rate as for positive animals (table 13).

Neuropharmacological evidence also gives support to the catecholamine hypothesis of self stimulation. Olds (1959) and Olds and Travis (1960) showed that self stimulation behaviour could be modified by drugs which interfered with some aspects of catecholamine metabolism, and Stein (1962) used the effects on self stimulation behaviour in rats, of the interactions of imipramine and amphetamine, and chlorpromazine and reserpine, to postulate a possible neurophysiological basis of depressive illness. He used a novel system, in which the rat was trained to press two levers. One lever worked conventionally, delivering to the animal a train of impulses of a particular current strength, but with the difference that successive stimuli were delivered at a progressively lower current strength. The second lever had the effect of resetting the current to the maximum level, but did not itself cause the delivery of a stimulus. Thus, animals could be trained to self stimulate until the current level was too low, when they would reset to the maximum. Individual animals showed definite behaviour profiles, resetting at the same current level

each time. Using this system, he noticed that chlorpromazine and reserpine raised the threshold for electrical reinforcement and in larger doses inhibited self stimulation entirely, whereas amphetamine lowered the threshold and increased the rate of self stimulation. The inhibition of chlorpromazine and reserpine was reversed by amphetamine. Imipramine acted differently to the other drugs. Given alone, it had a weak inhibitory effect, raising slightly the threshold at which resetting occurred. When given along with amphetamine, it potentiated the latter's effect. The importance of these results is two fold. Firstly, it firmly implicates catecholamines in the mechanisms of self stimulation behaviour and secondly, because of the clinical importance of these drugs in the treatment of affective disorders, suggests that self stimulation could reflect in some way the clinical state. On the basis of these results, Stein suggested that the depressed patient suffers from insufficient positive reinforcement, which might be due to a pathologically hypoactive neuronal system, or due to the inhibition of the system by the excessive activity of an anxiety-aversion system.

Similar ideas that depression was caused by the hypoactivity of an amino containing system in brain were put forward by Schildkraut (1965) and Ashcroft et al (1965) following the observation that groups of depressed patients showed a reduction in the concentration of 5HIAA, the major metabolite of 5HT, in lumbar CSF, when compared to neurological control patients (Ashcroft et al 1966). These

observations have been confirmed by other workers (Denker et al 1966, Van Praag et al 1970), and low levels of the major dopamine metabolite, homovannilic acid (HVA) have also been reported (Roos and Sjostrom 1969, Papeschi and McClure 1971). It might then be expected that recovery from depression would result in normal levels of metabolites in the CSF, but this has not been shown to be the case (Coppen et al 1972, Ashcroft et al 1973).

Since the realization that HMPG and not VMA was the major metabolite of cerebral noradrenaline attempts have also been made to study the concentration of this compound in CSF,urine and plasma in patients with affective disorders. Many conflicting reports have emerged from measurements in plasma and urine (Wyatt et al,1971; Nelson et al, 1966; Takahashi et al, 1968) because of the problem of assessing the peripheral contribution in these fluids. CSF levels, however, are thought to reflect brain levels, but measurements of HMPG in CSF (Wilk et al 1971, 1972, Bond 1972, Pullar 1973, Shaw 1973) have not shown any conclusive evidence to support the simple amine hypothesis 'that depression can occur when the levels of the amines at the reactive sites within the brain are reduced' (Ashcroft et al 1966), although in one study (Pullar 1973) the CSF levels of HMPG were significantly higher than controls in a group of manic patients and a group of agitated depressed patients.

In the light of neurophysiological results which show that the post synaptic cell may alter the firing rate of the presynaptic cell by neuronal feed-back inhibition

(Foote et al 1969) after direct receptor stimulation with the drug LSD, and information about changes in receptor sensitivity following denervation in the periphery (Sharpless 1964) and in the CNS (Ungerstedt 1971), Ashcroft et al 1972 have suggested a modified hypothesis for the aetiology of affective illness which accomodates the fact that patients with low CSF metabolites do not show normal levels on recovery, and which explains the paradoxical response of some patients to amphetamine, where the condition of hyperkinesia in children and two cases of hypomania could be controlled by this 'antidepressant' drug. The new hypothesis suggests as before that the activity of amine containing systems in affective illness is abnormal, but that this may take the form of an altered input into the neuronal systems from other centres, or might be due to an altered sensitivity of the post-synaptic receptors. Recovery would then be associated in depression with either a rise in receptor sensitivity or a rise in transmitter output, or both.

Supporting these ideas we have put forward an animal model which suggests a possible change in the post-synaptic receptor activity of the central noradrenergic pathway from the locus coeruleus to the cerebral cortex (Ashcroft et al 1973, Eccleston 1973). Numerous hormones (including noradrenaline) have been shown to act upon cells by stimulating the membrane bound enzyme, adenylatecyclase, which converts ATP to cyclic AMP, the latter giving rise to intracellular biochemical events. (Sutherland et al 1966,

Greengard and Kuo 1970). It has also been shown that rat brain cortical slices, when incubated with substrates in vitro, have a steady level of adenylatecyclase activity which can be increased by stimulating the slices with noradrenaline. Thus if slices are incubated with (^{14}C)-adenine, a precursor of (^{14}C) ATP, they are capable of converting some of the (^{14}C) ATP into (^{14}C) cyclic AMP (Shimizu et al 1969), the amount of cyclic AMP formed increasing with a concentration dependant relation to added noradrenaline (Eccleston 1973). When the locus coeruleus was unilaterally ablated, and the pathway from the LC allowed to degenerate over a period of 3 weeks, it was found that slices of cortex from the ablated side had almost double the capacity to form (^{14}C) cyclic AMP compared to slices from the intact side. Further, stimulation of the slices with noradrenaline caused an equivalent rise of cyclic AMP on both sides, so that slices of the ablated side still produced twice as much cyclic AMP as the intact side slices. This change in enzyme activity was absolutely correlated with the successful ablation of the homolateral locus coeruleus, checked for each experiment. Lesions placed around the nucleus had no effect on the adenyl cyclase system in cortex slices.

Thus, denervation of the NA-pathway from the locus coeruleus to the cerebral cortex by ablation of the locus coeruleus gives rise to a decrease in HMPG (table 15) but to an increase in adenylatecyclase activity. Thus a low metabolite level would not necessarily be associated with

a decreased activity of the post synaptic cell, but could even give rise to an increased activity.

Although the idea expressed by Stein (1962) and others, that depression could be explained by a lack of positive reinforcement, which seems to coincide to a certain extent with the fact that self stimulation is only associated with dopamine-containing and noradrenaline containing neuronal systems, Crow (1973) favours the view that this positive reinforcement behaviour is concerned more with learning than with the control of mood, and has suggested that because the pathways conveying information about smell and taste, (two very important factors associated with the motivation of the rat) are situated in an anatomical position so that they could interact with the DA and NA systems respectively, these latter systems could reflect two components of the 'exploratory drive' of the rat, an incentive motivational' component and a 'purely reinforcement' component respectively.

In summary, evidence is presented here that:-

a) the neuronal system with cell bodies in the locus coeruleus and terminal structures in the cerebral cortex is noradrenaline containing, since electrical stimulation of the locus coeruleus gives rise to an increase in the concentration of HMPG, the major metabolite of noradrenaline, in cerebral cortex.

b) the system is uncrossed, since unilateral ablation of the locus coeruleus gives a homolateral reduction of metabolite in cortex, without affecting the contralateral side.

c) there is an alternative pathway which does cross the mid line, since specific stimulation of one locus coeruleus gives rise to an increased concentration of metabolite on the stimulated and contralateral sides of cortex.

d) conscious electrical self stimulation in rats with electrode tips at the locus coeruleus is absolutely correlated with the activation of the LC-cortex neuronal system, shown by the parallel increase of HMPG in the cortex of positive animals which had histologically proven correct electrode tip positions.

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